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Differential expression in "rogue" paramutation in peas (*Pisum sativum* L.) - from mRNA to siRNA -

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Dissertação

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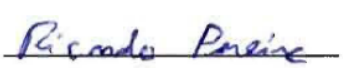

Ricardo Pereira

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Resumo

A paramutação é um fenómeno epigenético originado pela interação entre alelos que provoca alterações hereditárias na expressão génica. Embora estas interações sejam mais frequentemente observadas entre alelos do mesmo gene também existe casos onde as paramutações foram observadas entre sequências homólogas em posições não alélicas.

Os mecanismos moleculares que causam este fenómeno são ainda desconhecidos, no entanto em vários casos a paramutação tem sido associada à ação de RNAs não codificantes (ncRNA), a alterações na estrutura da cromatina e à metilação do DNA.

A metilação do DNA consiste na relocação de um grupo metil (CH3) de uma S-adenosil-l-metionina para o carbono 5’ da citosina ou adenina. Isto tem uma forte influência epigenética pois confere informação hereditária que não é codificada na sequência de DNA.

Os ncRNAs, que podem ser divididos em várias subcategorias tais como: micro-RNAs (miRNAs), long non-coding RNAs (lncRNAs), Piwi-interacting RNAs (piRNAs); enhancer RNAs (eRNAs), promoter-associated RNAs (PARs) e small interfering RNAs (siRNAs), têm sido associados a vários mecanismos que afetam a expressão génica, na regulação transcricional e pós-transcricional.

O primeiro caso de paramutação descrito foi o fenótipo Rogue em ervilheira (*Pisum sativum* L.), porém, os mecanismos moleculares responsáveis pelo aparecimento espontâneo e pela manutenção deste fenótipo não foram ainda esclarecidos, tal como não foram até ao momento esclarecidos, de forma inequívoca, todos os outros casos conhecidos de paramutação.

No entanto, sabe-se que o cruzamento de plantas Rogue com plantas do tipo selvagem resulta unicamente em plantas F1 "rogues" e que em todas as gerações seguintes as descendências são totalmente "rogue". A herança deste fenótipo encontra-se em contradição total com as regras de hereditariedade Mendeliana que preveem o aparecimento na geração F2 de pelo menos um quarto de indivíduos com o fenótipo selvagem e a duplicação desta percentagem em cada ciclo seguinte de autofecundação.

Este trabalho teve como objetivo avançar no caminho de descodificação deste fenómeno, aberrante do ponto de vista da genética clássica, tentando identificar diferenças de expressão génica entre uma cultivar de ervilheira (cv.Onward, JI2722) e

uma sua linha paramutada (Onward “Rogue”, line JI2723). Ambas gentilmente cedidas pelo Dr. Mike Ambrose do John Innes Institute, Reino Unido.

De acordo com esse objetivo procedeu-se à extração de RNA total de folhas jovens totalmente desenvolvidas de vários indivíduos dos dois tipos de plantas com a mesma idade e cultivadas em condições o mais idênticas possível. Do RNA procedeu-se ao isolamento do RNA mensageiro a partir do qual se procedeu à síntese de cDNA monocatenário, utilizado para realizar a maioria dos trabalhos efetuados para a preparação desta dissertação.

A fim de procurar diferenças na expressão génica efetuaram-se vários ensaios de Multi-RAPD Differential Display (MRDD), cuja técnica é baseada na técnica do Differential Display mas com algumas diferenças nomeadamente, omitindo o oligo-dT e substituindo a utilização de um único primer RAPD por uma combinação de quatro primers do mesmo tipo. Foram testadas 109 combinações diferentes de 4 primers RAPD, no entanto entre os mais de 700 produtos de amplificação nenhum se apresentou como polimórfico. Alguns resultados prévios que apontavam para a possível existência de polimorfismos não foram confirmados em segunda análise utilizando outras amostras biológicas.

A procura de polimorfismos na expressão génica foi então direcionada para a confirmação de expressão diferencial de sequências indicadas como estando presentes em quantidades diferentes nas amostras de cDNA da cv. Onward e da sua linha paramutada (Rogue) pela análise por Suppression Subtractive Hybridization (SSH) previamente efetuada pelo Laboratório.

Entre os inúmeros contigs fornecidos pela sequenciação massiva paralela (next generation sequencing) das bibliotecas geradas pela análise SSH (Santo e Leitão, resultados não publicados) foram selecionados 24 para análise neste trabalho. Desenharam-se primers que flanqueiam pequenos fragmentos (95 a 120 bp) dos 24 contigs, passiveis de serem analisados com alta eficiência pela técnica de RT-qPCR.

No entanto, tendo em linha de conta a grande quantidade de sequências aparentemente expressas diferencialmente identificadas pela técnica SSH, e os maiores requisitos em tempo, recursos humanos e materiais, da técnica de RT-qPCR, procedeu-se à avaliação prévia, mas menos precisa, da expressão diferencial das 24 sequências pela comparação dos produtos da amplificação com menos ciclos (25 ciclos) por PCR. A validação deste teste prévio, teve como objetivo estabelecer um procedimento de

seleção que permita dar prioridade na análise por RT-qPCR às sequências com maior probabilidade de apresentarem diferenças significativas de expressão. Procede-se em paralelo a amplificações por 35 ciclos que serviram de controlo aos resultados das amplificações por 25 ciclos.

A comparação dos resultados por amplificação por RT-qPCR e os resultados da amplificação prévia por 25 ciclos de PCR demonstrou uma forte correlação entre estes dois tipos de análise, o que permitirá focar as análises por RT-qPCR nas sequências melhores candidatas a apresentarem diferenças significativas na sua expressão.

Os resultados da análise RT-PCR permitiram identificar diferenças na expressão de 11 sequências, das quais 8 com diferenças significativas e que poderão estar associadas a mecanismos moleculares relacionados com a paramutação. No entanto estes resultados terão de ser confirmados utilizando amostras biológicas adicionais.

Estudos recentemente desenvolvidos no Laboratório (Santo e Leitão, resultados não publicados) provaram a existência de metilação diferencial de sequências genómicas específicas nas folhas e no pólen de plantas da cv. Onward e linha Rogue. Estes dados chamam a atenção para os genes relacionados com metilação de DNA e modificações da cromatina e para possíveis alterações na sua expressão no processo de paramutação. Não sendo conhecida a sequência destes genes em *Pisum sativum* torna-se necessário identificar, pelo menos de forma parcial, a sequência exata de alguns desses genes nesta espécie antes de se proceder à análise da sua expressão dos mesmos em plantas rogues e não-rogues. Com base nos dados genómicos referentes a esses genes em *Medicago truncatula* (http://www.jcvi.org/medicago) foram sintetizados primers que permitiram amplificar algumas regiões dos genes *ddm1, drm2* e *mop1* em ervilheira, que foram confirmadas com elevada similaridade com as sequências dos mesmos genes em *M. truncatula* e *Cicer arietinum* (grão de bico) e em particular com elevada similaridade das sequências proteicas que estas codificam (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Estas sequências serão utilizadas em breve para avaliar a expressão diferencial destes genes em plantas paramutadas (Rogue) e não-paramutadas (cv. Onward) de ervilheira.

A paramutação tem sido associada a mecanismos de metilação de DNA dirigidos por RNA (RNA directed DNA methylation - RdDM) pelo que demos inicio ao estudo comparativo das classes de siRNA presentes em plantas paramutadas e não-paramutadas. Com esse objetivo procedeu-se ao isolamento de siRNA em plantas

Onward e Rogue, separando o RNA total em duas frações distintas (alto peso molecular e baixo peso molecular) por precipitação fraccional com PEG e NaCl e à ligação de adaptadores RNA à fração de baixo peso molecular. O cDNA resultante dos siRNA ligado aos adaptadores foi amplificado por PCR durante 35 ciclos e durante 6 ciclos, respetivamente para visualização e para excisão (recuperação) em gel de agarose. A região entre os 71 e 76 bp (siRNA mais adaptadores) foi purificada para futura validação e posterior analise após sequenciação massiva paralela.

No entanto, o estudo da expressão génica diferencial por via da análise das sequências previamente identificadas pela técnica de Suppression Subtractive Hybridization (SSH) parece ser bastante prometedor e passível de identificar alterações de cadeias metabólicas associadas ao estabelecimento e manutenção da paramutação, pelo que se apresente como prioritário e urge ser rapidamente continuado.

Abstract

The "rogue" phenotype in peas (*Pisum sativum* L.) was the first reported case of paramutation, however, since this finding, most of the studies focussed on some cases of paramutation in maize. The main aim of this work was the identification of differentially expressed tags in the pea cv. Onward vs. its paramutated rogue line JI2723. One hundred-nine combinations of 4 RAPD primers were used in multi-RAPD differential display analysis, but no expression polymorphisms were identified between the two epigenomes. However, the RT-qPCR analysis of 24 out of the over 120 putatively differentially expressed sequences identified via next generation sequencing of suppression subtractive hybridization (SSH) libraries, allowed the identification of 11 differently expressed sequences. Among these sequences, 8 exhibited very significant differences in their expression in the two epigenomes. A procedure for pre-selection of putatively differently expressed sequences before more accurate confirmation by RT-qPCR analysis was developed and is expected to increase the efficiency of the analytical procedure. Recent studies performed in the Laboratory proved the existence of differences in the DNA methylation between the two epigenomes. Partial sequences of the genes related with DNA methylation and chromatin remodelling, *ddm1*,*drm2* and *mop1* were retrieved from the pea genome permitting the expression of these genes to be analysed in the paramutated vs. non-paramutated epigenomes. siRNA libraries of the two epigenomes are under construction and are expected to allow the identification of specific classes of siRNA associated with the "rogue" paramutation.

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1-Introduction

1.1-Epigenetics

The term “Epigenetics” was defined by Conrad Waddington (1905-1975) as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” [1]. But this concept, like others, evolves and epigenetic can be also defined as “how genotypes give rise to phenotypes during development” or “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” [2].

In spite of the different definitions, the objective of epigenetics is the study of mechanisms of gene regulation and mitotical and meiotical inheritance of information not encoded by the DNA sequence. Therefore epigenetics is responsible for establishing a connection between genotype and phenotype[3][4][5][6].

Observing a multicellular organism all cells are genetically similar but due to the differential expression these cells present very distinct structures and functions. The difference between the expression of cells is said to be epigenetic [3]. Usually the epigenetic mechanisms that are the cause of this difference in gene expression are DNA methylation, modifications of the chromatin and the action of non-coding RNAs [1][7].

1.2- DNA methylation

DNA methylation consists on the relocation of a methyl group (CH_3) from S-Adenosyl-L-Methionine (SAM) to the carbon 5’ in the cytosine or adenine. This addition has a strong epigenetic influence as it confers inheritable information that is not encoded in the DNA sequence [8].

In eukaryotes, DNA methylation can influence a large range of biological functions such as gene expression, regulation of development, conservation of genome integrity and inactivation of X-chromosome in mammals. In prokaryotes it also has a great impact in biological functions such as differentiate self and non-self DNA and to coordinating DNA replication and the cell cycle [8].

In mammals, DNA methylation is almost restricted to occur in repeated regions of cytosine and guanine dinucleotides, it is belived that 70-80% of CG dinucleotides in these genomes are methylated, however there are regions normally found near gene promoters where is possible to find unmethylated CG dinucleotides in the CpG islands. In plants, DNA methylation usually occurs at cytosine bases within all sequence contexts: the symmetric CG and CHG contexts (where H=A, T, or C) and the

asymmetric CHH context. Genome wide, DNA methylation levels of approximately 24%, 6.7% and 1.7% are observed for CG, CHG, and CHH contexts, respectively. [9].

As mentioned before DNA methylation is a factor with high importance in gene regulation, typically presenting a repressive effect in transcription. This repressive effect occurs at three levels of control: 1) Several transcription factors, like AP-2, c-Myc/Myn, E2F, and NF"B, are not able to bind to methylated target sites. 2) DNA methylation recruits 5-methylcytosine binding proteins that act as repressors of gene transcription. 3) DNA methylation triggers histone deacetylation and thereby induces chromatin condensation which leads to a strong and stable repression of gene expression.[8].

1.3- Non-coding RNAs

Non-coding RNAs (ncRNAs) can be divided in subcategories, like micro-RNAs (miRNAs), long non-coding RNAs (lncRNAs), Piwi-interacting RNAs (piRNAs); enhancer RNAs (eRNAs), promoter-associated RNAs (PARs) and small interfering RNAs (siRNAs). These RNAs have been associated with a variety of mechanisms that modulate gene expression and the mitotic, meiotical and transgenerational inheritance of epigenetic signals can be accomplished in part by non-coding RNAs [10][11][12].

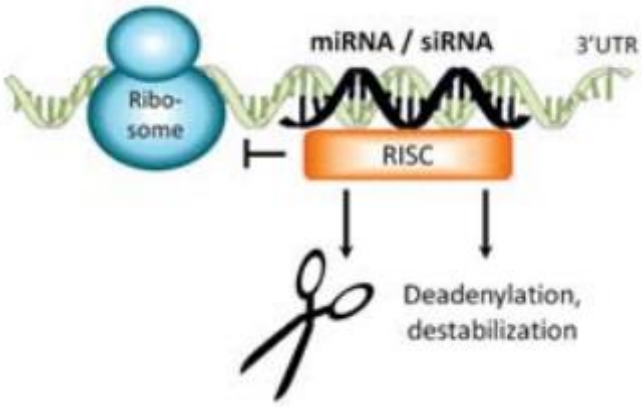

Figure 1 Post-transcriptional gene silencing mechanisms mediated by siRNAs and miRNAs, which are incorporated into RNA-induced silencing complexes (RISCs) that target specific mRNAs for cleavage, translational repression or destabilization (adapted from [10]).

Multiple ncRNAs have been established as negative transcription regulators and also playing a role in post-transcriptional regulation, like splicing, transport, translation, and degradation. One example of these mechanisms is the post-transcriptional gene silencing (PTGS) mediated by siRNAs and miRNAs (Fig. 1) where the two types RNAs

affect the expression of genes by influencing the stability and the translation of mRNA, the siRNAs silence the loci they are resulting from, whereas the miRNAs regulate other genes [10].

The effects of ncRNAs in transcription regulation has become an area of great interest and research, but the involvement of these RNAs in the regulation of expression is still far from being completely understood [10].

1.4- Paramutation

Paramutation is an epigenetic phenomenon in which an epigenetic state of an allele is transferred to another allele in trans, resulting in a heritable modification of the gene expression of the second allele [13][14]. While the interactions are most frequently observed between alleles of the same gene, paramutations have also been observed between homologous sequences at non-allelic positions [15][16].

By definition the allele that induces an epigenetic change on the other allele is considered to be paramutagenic and the sensitive allele is paramutable, while alleles that do not participate in the paramutation are designated as neutral. Frequently, the modified (paramutated) alleles also becomes paramutagenic, acquiring the ability of alter other sensitive alleles [13][17].

This phenomenon can be characterized by tree basic characteristics: i) the new epigenetic state is transferred to the following generations even if the original allele that induced the new epigenetic state is not transmitted; ii) the altered allele also acts like the paramutagenic allele; and iii) there is not any associated alteration in the DNA sequence[18].

It is still not understood how the paramutation occurs, but there are models that have already been proposed to explain the trans communication between alleles during paramutation like: i) the "trans RNA model" where the communication is mediated by intermediary RNA (siRNA and ncRNA) molecules; and ii) the "pairing model" that suggests that epigenetic states are altered by direct physical interaction between the intervening sequences. According to the current understanding of paramutation this models are not exclusive and can coexist and work together[17].

1.5- Paramutation in mouse *Kit* gene

Paramutations occurs both in animals and plants. One of the better studied cases of paramutation in mammals is the paramutation at the locus *kit* in mouse. The mouse *Kit* gene encodes the *Kit* tyrosine kinase receptor, fundamental in several processes

during mouse development such as germ cell differentiation, melanogenesis and haematopoiesis. By inserting a 3-kilobase (kb) lacZ-neo cassette downstream of the initiator site it was possible to engineer a null mutant *tm1Alf* [19].

The *tm1Alf* mutation annuls the synthesis of the kit tyrosine kinase receptor and the Kit^{tm1alf} homozygotes die shortly after birth. The heterozygotes (Kit^{tm1Alf}/Kit^{+}) comparing to the wild-type mouse present a different phenotype, white tail tip and white feet (Fig. 2), due to the reduced level of expression of the *Kit* receptor [19].

Figure 2 Heterozygote mouse, genotype and phenotype (adapted from [19])

Comparing the levels of *Kit* mRNA from the mutant phenotype and the wild-type, it was determined that the mutant phenotype mouse has one-half of the *Kit* mRNA found in the wild-type. When heterozygous Kit^{tm1Alf}/Kit^{+} are crossed or intermated with wild-type homozygous (Kit^{+}/Kit^{+}) most of the homozygous for the allele wild-type reveal the same phenotype and lower levels of kit mRNA as the heterozygous parent, which suggests that Kit^{tm1Alf} is paramutagenic and the wild allele has adopted a new epigenetic state denominated Kit^{*} (the wild allel was paramutated) [19].

The microinjection of fertilized eggs with either total RNA from Kit^{tm1Alf}/Kit^{+} heterozygotes or Kit-specific microRNAs induced the white tail phenotype, evidencing the role of RNA in *Kit* paramutation [19].

1.6- Paramutation at b1 locus in maize

One of the most extensively studied cases of paramutation is the *b1* locus in maize. The *b1* locus is related with anthocyanin synthesis. There are several alleles for this locus, but only two participate in the paramutation phenomenon, *B’* and *B-I*. Like described for all paramutation systems, both epiallels have the same DNA sequence, yet the B-I expression level is much higher than the B ' [7]. When *B-I* plants are crossed with *B’* plants the heterozygous *B’*/*B-I* plants behave as homozygous *B’*/*B’*, the epiallel *B-I* is converted to the epigenetic B’ state and becomes paramutagenic [17]. Located 100 Kb upstream of the locus b1, both epialleles have seven tandem repeats of 853 bp absolutely obligatory for paramutation to occur since alleles with only one copy of the repeated sequence are non-paramutable. Although absolutely identical in what concerns the DNA sequence the repeats are differently methylated and show differences in sensitivity to DNaseI [17][20].

1.7- Rogue phenotype in *Pisum sativum L*

The occasional emergence of plants exhibiting a Rogue phenotype with pointed leaflets and leaf stipula among self-fertilized pea (*Pisum sativum* L.) lines(Fig. 3), and the extraordinary inheritance of these new traits, which not abide by the Mendelian rules, were described for the first time in the early 20th century [21].

Figure 3 “Rogue” phenotype in Pisum sativum L. 1) spontaneous “rogue” mutant cv. Onward plant.; 2) cv. Onward plant

As for the other later described paramutation cases, the offspring of self-fertilised “rogue” (paramutated) plants consist only in “rogue”, and when crossed with wild types these plants produce uniquely F1 plants that as they develop turn into “rogues”. All plants in the following generations are also “rogue”.

1.8- Genes involved in DNA methylation, chromatin remodeling and paramutation

Multiple genes have been identified involved in DNA methylation, chromatin remodelling and paramutation. Three out of these genes are *ddm1, drm2* and *mop1*.The *ddm1 gene* is involved in DNA methylation particularly in small-RNA-directed DNA methylation (RdDM) mechanism. The product of this gene belongs to the family of Snf2 remodelers that allow other proteins to access the DNA by changing the nucleosome placement and composition**.** Recent studies showed that *ddm1* is involved in the stable silencing of transposable elements by allowing DNA methyltransferases in collaboration with RdDM to interact with histone H1-containing heterochromatin [23].

The gene *drm2* (domains rearranged methyltransferase 2) is associated with the maintenance of non-CG methylation and also with *de novo* DNA methylation. Mutations in *drm2* can block all *de novo* DNA methylation associated with repeat containing transgenes. The *drm2* function is associated to small RNAs since they are essential to maintain non-CG DNA methylation and site-directed mutagenesis tests have shown that the RNA-directed DNA methylation is dependent of both the ubiquitin-associated (UBA) and catalytic methyltransferase domains of *drm2*. [23][24].

The gene modifier of paramutation 1 (mop1) encodes an RNA-dependent RNA polymerase [25], which when mutated allow the low-expressing paramutagenic allele (*B'*) to express identically to the highly expressing paramutable allele (*B-I*), preventing the paramutation of a new *B-I* allele. These facts indicate that the expression of mop1 is generally necessary for paramutation, however mutations in this gene affect multiple loci modulated by different promoters evidencing that the product of this gene probably operates on chromatin configuration and not on specific sequences [26].

2- Materials and Methods

2.1- Plant material

Seeds of Pisum *Sativum* L. cv. Onward (Line JI2722) and its paramutant Onward "rogue" line JI2723 were quickly washed with tape water and Tween-20 and immediately immersed for 5 minutes in disinfecting solution containing 10% bleach and 0,5% SDS, rinsed with tape water and germinated over moisten paper in petri dishes for 72 hours, at 24°C in the dark. The originated seedlings were transplanted to pots containing 1:1 peat: vermiculite mixture inoculated with macerated Rhizobium nodules, and grown in a greenhouse.

2.2- RNA extraction

RNA was extracted from fully expanded young leaves using Ribozol™ RNA extraction reagent according to the manufacturer protocol. Briefly, leaves were ground in a mortar with a pestle in the presence of liquid nitrogen and the resulting fine powder was thoroughly mixed with 9 volumes of Ribozol™. After 5 min agitation the homogenate was centrifuged at 4500rpm for 20 minutes at 4ºC to remove insoluble material, the supernatant was transferred to a fresh tube and incubated at room temperature for 5 minutes. Then, 200uL of chloroform per 1 mL of Ribozol™ were added followed by an incubation of 3 minutes at room temperature. The sample was centrifuged at 4500rpm for 20 minutes at 4ºC, the upper aqueous phase was transferred to a RNase-free tube. The RNA was precipitated by adding three volumes of 100% ethanol and stored at -80ºC.

2.3- mRNA isolation

mRNA was isolated with the “PolyATtract® mRNA Isolation Systems IV” kit according to the protocol provided by the manufacturer. Total RNA was resuspended in 500 µl Rnase free water in two sterile RNase-free 1.5ml tubes one containing 290,624 µg of Onward and the other containing 393,024 µg of Rogue and heated at 65ºC for 10 minutes, 3 µl of biotinylated-oligo(dT) probe and 13µl of 20X SSC were added to the warm RNA solution and the samples were cooled down at room temperature. One tube of the Streptavidin Paramagnetic Particles (SA-PMPs) per isolation was resuspended by gently flicking the bottom of the tube until they were completely dispersed. The SA-PMPs were captured by placing the tube in the magnetic stand. The supernatant was carefully removed after 3 washes with 300µl 0.5X SSC, using the magnetic stand between washes, the SA-PMPs were resuspended in 100µl of 0.5X SSC. The hybridized RNA was then added to the tube and incubated at room temperature for 10 minutes and gently mixed by inversion every 1–2 minutes. The SA-PMPs were captured using the magnetic stand and the supernatant was carefully removed. The particles were washed four times with 300µl 0.1X SSC and resuspended in 100µl RNase-free water. The eluted mRNA was transferred to a sterile RNase-free tube and stored at -80ºC.

2.4- cDNA first strand

The cDNA first strand was synthesized using the “Thermo Scientific RevertAid First Strand cDNA Synthesis Kit”. The reaction was made in 20 µl reaction mixture consisting of 1x Reaction Buffer, 1 U of RiboLock RNase inhibitor, 1 mM of each

dNTP, 5 µM mix of RT primers (Table 1), 10 U of RevertAid M-MuLV RT and 230 ng of mRNA. The reaction was incubated in a thermocycler (VWR) at 42ºC for 1 hour and terminated by heating at 70ºC for 5 min and stored at -80ºC.

Table 1. Mix of reverse transcription (RT) primers with respective primer sequences where N represents any base

	Primer name	Sequence
RT primers	T12AN	TTTTTTTTTTTTAN
	T12CN	TTTTTTTTTTTTCN
	T12GN	TTTTTTTTTTTTGN

2.5- Quantification of RNA, DNA and cDNA

All RNA and DNA samples were quantified using a Nanodrop 2000 eSpectrophotometer from Thermo scientific.

2.6- Multi-RAPD differential display analysis

Multi-RAPD differential display amplifications were performed using 109 different combinations of four RAPD primers (Operon technologies) (Annex I), previously tested for possible dimers (FastPCR version 4.0.27).

The PCR amplifications were performed in 15 µl reaction mixtures consisting of 1x Dream Taq Buffer (Fermentas), 0.16 mM of each dNTP, 0.5 µM of each primer, 0.6 U of Dream Taq DNA polymerase (Fermentas) and 15 ng of First Strand cDNA. The thermocycler (Biometra TGradient) was programmed as follows: 1 minute and 30 seconds initial denaturation cycle at 94ºC followed by 5 cycles of 30 sec at 94ºC, 30 sec at 36ºC, 1 min at 72ºC, and 30 cycles of 30 sec at 94ºC, 30 sec at 40ºC, 1 min at 72ºC, ending with an extension cycle of extension of 10 min at 72ºC. The amplification products were electrophoresed on 2% agarose gels. Gels were stained with ethidium bromide and photographed under UV trans-illumination with a digital camera "Kodak EDAS 120".

2.7- Low (25) cycles PCR and RT-qPCR amplifications

Twenty-four DNA sequences out of two large groups of sequences putatively differentially expressed in both epigenomes (cv. Onward and "rogue line JI2723) previously identified by the Laboratory (Santos and Leitão, unpublished results) via next generation sequencing of Suppression Subtractive Hybridization libraries, were

selected for further confirmation by low (25) cycles PCR and RT-qPCR. Primers were designed using the programme FastPCR (version 4.0.27) according to following requirements: no dimer formation, 17-21 base length, 50% - 60% GC content and ~50ºC melting temperature. All primers were ordered from NZYTech (Table 2).

Table 2 Primers for PCR and RT-qPCR amplifications of specific sequences

Name	Sequence	Ta°	Name	Sequence	Ta
R_Seq 8 Fw	TCTCCTTCATGGAGGTC	58	O_Seq 3 Fw	ATGGAGCACCAAGATATG	56
R_Seq 8 Rv	AACACGTCAAGGACTCT		O_Seq 3 Rv	AGATACAGAGATCAACCTC	
R_Seq 11 Fw	TGACAACTTGCCTATGG	55	O_Seq 5 Fw	CAGCAGTGATAGCCATAG	58
R_Seq 11 Rv	ACTGATAAGGGCATCTC		O_Seq 5 Rv	TGATTGAGAAGGCAACAC	
R_Seq 12 Fw	GAGTGGGACAGATTCAG	58	O_Seq 6 Fw	TCATTCTCCAAGGTTGCTG	58
R_Seq 12 Rv	TCAGCATCAATGTGACC		O_Seq 6 Rv	GGATACCTATCACCTAGAAC	
R_Seq 13 Fw	TCATGCGGAGGACTATC	58	O_Seq 7 Fw	TTCTTCAGGTGTGCAAC	58
R_Seq 13 Rv	CACCTTCCAAGCAAGG		O_Seq 7 Rv	TCCTGGTTGTCGATACTT	
R_Seq 14 Fw	TCCACAGCAATTCTGTG	55	O_Seq 8 Fw	TGAATTGCACTCCATCTC	58
R_Seq 14 Rv	AAGACATTCTCTGGCAAC		O_Seq 8 Rv	ATCCACTTTCTCCACTAC	
R_Seq 15 Fw	AGACACAACTTGGATCC	58	O_Seq 9 FW	TCAGCTCCAATTCTCCA	58
R_Seq 15 Rv	AATCGGTTGATCCTCAG		O_Seq 9 Rv	GCTTGCCAAATGGATC	
R_Seq 16 Fw	TCCTCTAACTCTTCAAGCA	58	O_Seq 10 Fw	AGTTCCTCGTAATCAGTGT	58
R_Seq 16 Rv	TATGACTGTGGAAATGGAAG		O_Seq 10 Rv	TTCTTGCATCTAGAGCTC	
R_Seq 17 Fw	CTGCTGTTGATGATATTG	58	O_Seq 11 Fw	ACATCTTCAATAGTTCCAAC	54
R_Seq 17 Rv	TTAGCCTTAGAAGAAGC		O_Seq 11 Rv	ATACACCACTGTTTATGTTG	
R_Seq 18 Fw	ACAACAGACGGTCATTG	58	O_Seq 12 Fw	TTGGTTGAACAAGCTTC	58
R_Seq 18 Rv	AATCGCTTCGGAAACTG		O_Seq 12 Rv	GTCTCAACAACCAGATC	
R_Seq 19 Fw	TCTGCCATCGAGATATCA	56	O_Seq 13 Fw	TAAGGTTGACCGTGTG	58
R_Seq 19 Rv	GTTCGCCTTTAACCAAG		O_Seq 13 Rv	TGGCTCCTGCATAATG	
R_Seq 20 Fw	AATGATAGACATGGCAGATG	58	O_Seq 14 Fw	TTCTGGATTGTTTGAGGA	58
R_Seq 20 Rv	AACAACCTGGCTTTGAG		O_Seq 14 Rv	TAACCAACTGAGCAACT	
O_Seq 2 Fw	ATCTGCATCTGATTGTG	56	O_Seq 15 Fw	ATTCGAGAAGGTATAGCATG	58
O_Seq 2 Rv	CTCTGAATTATCAACTACAGA		O_Seq 15 Rv	TAGTAGGCATGGTCAGA	

Low (25) cycles and typical (35 cycles) PCR amplifications were performed and analyzed in agarose gels as above described.

2.8- Real-time PCR

Real-time PCR amplifications were performed in 15 µl reaction mixtures consisting of 2x iQ™ SYBR® Green supermix, 0.5 µM of each primer, and different dilutions of the First Strand cDNA. The reaction was performed in a iCycleriQ® Multicolor Real-Time PCR Detection System using the following program: 5 minute of initial denaturation at 95ºC followed by 35 cycles of 10 sec at 95ºC, 30 sec at 56-58ºC

annealing temperature, where the data collection was enabled, followed by 1 cycle of 1 minute at 95ºC and um cycle of 1 minute at 55ºC ending with 80 cycles of 10 seconds starting with 55ºC where the setpoint temperature is increased by 0,5ºC after cycle 2 and the melt curve data collection and analysis becomes enabled.

2.9- Identification of homolog sequences of the *ddm1*, *drm2* and *mop1* genes in *Pisum sativum* L.

Primers for amplification of the still unidentified homolog sequences of the *ddm1*, *drm2* and *mop1* genes in *Pisum sativum* L**.** (Table 3) were designed using as reference the *Medicago truncatula* genome information (http://www.jcvi.org/medicago), additional information previously obtained in the lab and the *Pisum* unigene database in http://www.coolseasonfoodlegume.org.

The PCR amplifications were performed as above described except for the reaction volume that was scaled-up to 30 µL. Five microlitters of the amplification reactions were used for agarose gel analysis and in the cases where one single PCR product was obtained the remaining (25µL) amplification product was precipitated with 3 volumes of absolute ethanol and the dried pellet send for sequencing. In the cases were more than one band was amplified, the band with the expected length was excised from the gel, purified using the "Thermo Scientific GeneJET Gel Extraction Kit #K0692" and sent for sequencing.

Table 3 Primers for isolation of ddm1, drm2 and *mop1* gene sequences in *Pisum sativum*

Primer name	Primer sequense	bp	Annealing temperature (ºC)
DDM1exp_F6	AAGAACAATGTGAAGAACGA	617	55
DDM1exp_R5	TCAGCAAGAATCCCATTC		
DDM1_F10	TGCCTTTACTAACTGGTGG	989	55
DDM1 exp R4	GCATTGCTCAATTATCTC		
DRM2exp_F9	TGGTTGATACAATTGGAGAG	600	55
DRM2exp_R6	GTGAGGAGTTAGGACCT		
MOP_FW1p	TGAAGAAGCATATGATCATCAAC	407	55
MOP_Rv3p	CAGACTTGATATGCAATAAAATGTC		

2.10- Isolation and preparation of siRNA libraries for next generation sequencing

To total RNA in RNase-free water (*Pisum sativum*, cv. Onward and its paramutant "rogue" line JI2723)) were added 50% PEG (MW=8000) to a final concentration of 5%

and 5M NaCl to a final concentration of 0,5M for precipitation of the high molecular weight (HMW) RNA [27]. After centrifugation at 17000 rpm for 20 minutes at 4ºC and the supernatant containing the low molecular weight (LMW) RNA was precipitated overnight with tree volumes of ethanol 100%.

The LMW RNA was collected by centrifugation at 17000 rpm, for 30 minutes at 4ºC, and the pellet dissolved in 10µl DEPC-treated water. The ligation of 3' RNA adaptor was carried out incubating for 1 hour at 37ºC in a reaction mix with 10 µl consisting of: 5 µl of LMW RNA solution previously denatured at 70ºC for 15 minutes, 2 µl of 3' RNA adaptor from biomers.net (20µM stock concentration), 1 µl 10x RNA ligase buffer and 2µl T4 RNA ligase.

Three microliters of (100 µM) RT 3'- primer (Table 4) were added to the 3' RNA adaptor ligated LMW RNA and the mix incubated at 75ºC for 5 minutes, followed by 37ºC for 15 minutes and 25ºC for 15 minutes. To this reaction mix were added 2 µl of (20µM) 5' RNA adaptor denaturated at 70ºC for 15 minutes, 1 µl 10x RNA ligase buffer, 2µl T4 RNA ligase, and the ligation of the 5' RNA adaptor was carried out for 1 hour at 37ºC. The resulting LMW RNA ligated to both adaptors and annealed to the RT primer was then used for first strand cDNA synthesis as above described.

The obtained cDNA was used for two different PCR amplifications using the 5'-primer (Table 4) and the above mentioned RT 3'-primer: i) 6 cycles of amplification for synthesis of cDNA second strand and low amplification of the ligated products, for further next generation sequencing; and ii) 35 cycles for amplification of and easily scored in 10% polyacrylamide/urea gel product. The amplifications were performed in 20 µl reaction mixtures consisting of 1x Phusion HF buffer (Thermo scientific), 0.16 mM of each dNTP, 15 µM of each primer, 0.4 U of Phusion DNA polymerase (Thermo scientific) and 1 µl of cDNA, in a thermocycler (Biometra Tgradient) programmed for: starting denaturation at 94ºC during 1 minute and 30 seconds followed by 6 or 35 cycles of 30 sec at 94ºC, 30 sec at 62ºC, 1 min at 72ºC and one cycle of 10 min at 72ºC.

Table 4 Primers used in the siRNA isolation and amplification

Primer name	Primer sequence
siRNA PCR Primer	GTC TAG TCG CAT CCT GTA GA
siRNA RT-PCR Primer	GCA GGT GTC AGC ATC AGT CTG CAT A

3- Results and discussion

3.1- Extraction of total RNA and mRNA isolation.

The extraction of total RNA of *Pisum sativum* L. cv. Onward (Line JI2722) and Onward “rogue” (Line JI2723) was performed using the Ribozol™ RNA extraction reagent according to the manufacturer protocol. The extraction was performed using 9 ml of extraction reagent to which the fine powder resulting from leaves grounded in a mortar with a pestle in the presence of liquid nitrogen was added until the total volume reached 10 ml.

From 1 ml volume grounded leaf tissue powder of cv. Onward and Onwrd “rogue” line were isolated , respectively, 581,25 µg and 786,05 µg total RNA. The integrity of the extracted RNA from both epigenomes was confirmed by agarose gel electrophoresis the samples were mixed with an equal volume of formamide (Fig 4). The above amounts of total RNA resulted, respectively, in 400 ng of mRNA of cv. Onward and 720 ng mRNA of the “rogue” line, isolated using the “PolyATtract® mRNA Isolation Systems IV” kit. The mRNA of both epigenomes was used for the synthesis of single stranded cDNA which concentration was normalised between both samples and used for differential expression analysis.

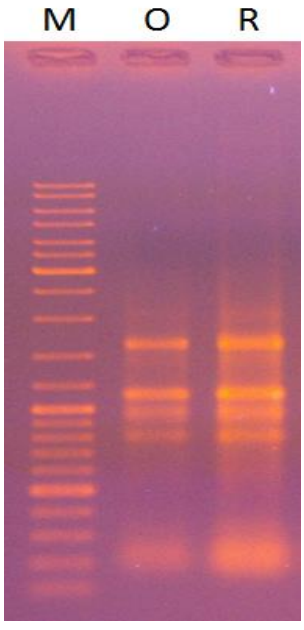

Figure 4 Total RNA from: (O) cv. Onward and (R) “rogue” line JI2723.

3.2- Multi-RAPD differential display analysis

The differential display technique is commonly used for identification of expression polymorphisms between two or more organs or organisms using a short arbitrary primer in combination with an anchored oligo-dT primer.

In this work the technique was modified using a new approach which we have designated Multi-RAPD Differential Display (MRDD). The main difference in this technique is that the oligo-dT is omitted and the amplification process is performed using combinations of four RAPD primers in order to increase the odds of polymorphism identification. The PCR amplifications are carried out in two steps: i) a first step using an annealing temperature of 36 °C for 5 amplification cycles and ii) a second step of 30 cycles during which the annealing temperature was increased to 40 °C One hundred and nine combinations (Annex I) of 4 RAPD primers were used for identification of expression polymorphisms between cv. Onward (Line JI2722) and its paramutant "rogue" line (JI2723). Although, some very few putative polymorphisms were identified in a first round of analysis, they were not confirmed in a second round of analysis with other biological samples. Among the over 700 amplified expression markers no one was polymorphic. (Fig 5 and Fig 6).

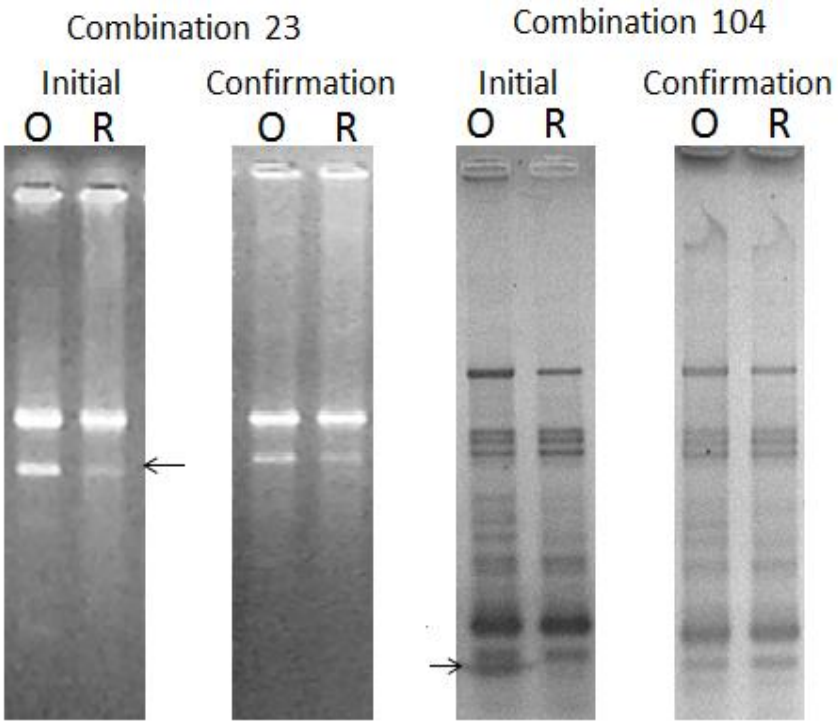

Figure 5 Example of two combinations of Multi-RAPD differential display patterns that presented differences, but were proven to be false after repeating the tests with different biological samples. (O) cv. Onward; (R) "rogue" line JI2723.

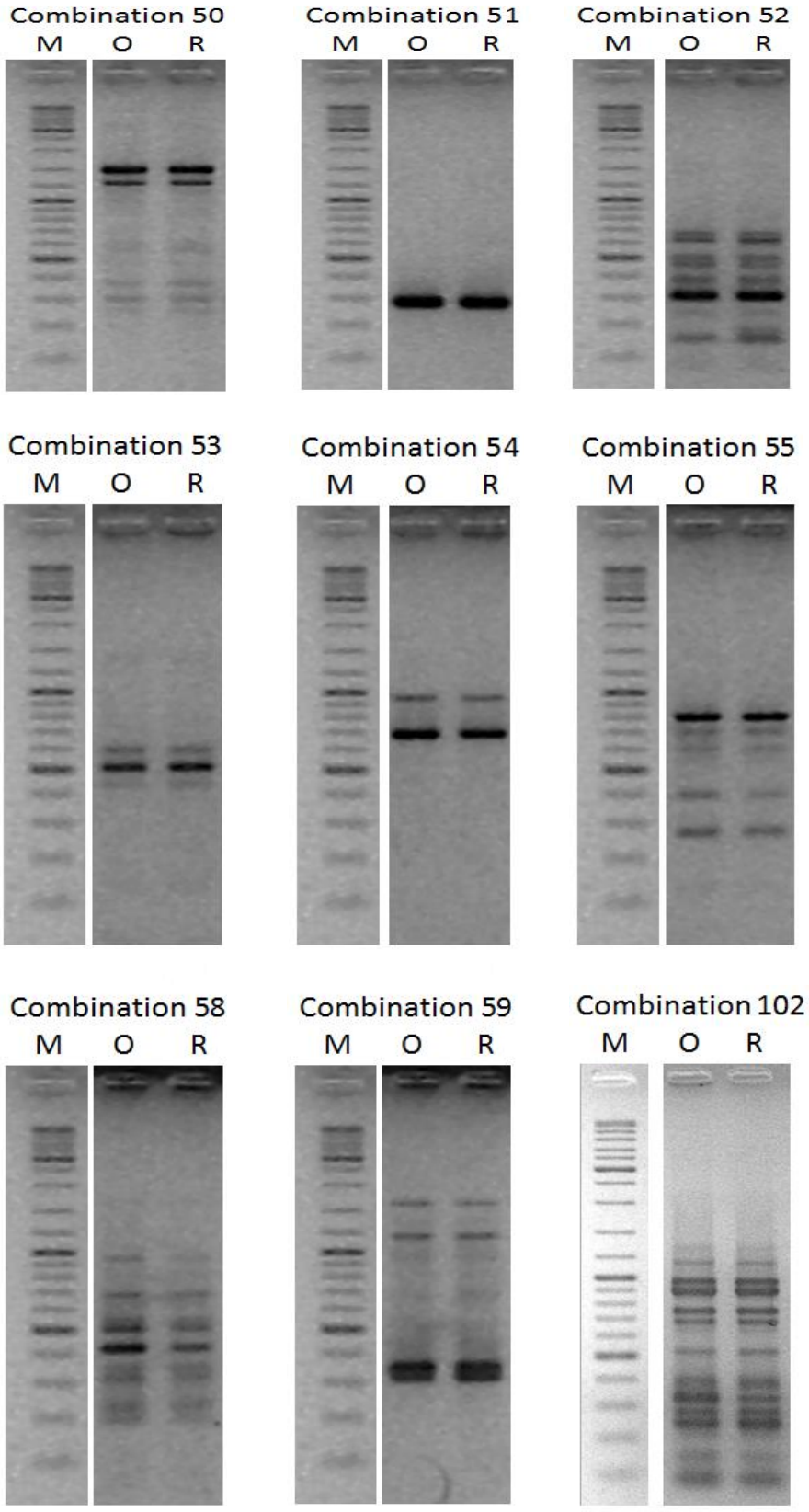

Figure 6 Examples of Multi-RAPD differential display patterns that did not present differences. (O) cv. Onward; (R) “rogue” line JI2723 (M) Marker, Thermo scientific GeneRuler DNA Ladder Mix.

3.3- Expression analysis of SSH-selected sequences

Since no positive results were found using the multi-RAPD differential display analysis the search for differential expressed sequences between the two *Pisum* epigenomes was continued focusing on the confirmation of the differential expression of the expressed sequence tags (ESTs) identified via next generation sequencing of the contrasting Suppression Subtractive Hybridization (SSH) [28] libraries of cv. Onward and Onward “rogue” line JI2723 (Santo T and Leitão J, unpublished data).

The expression analysis was performed in a two steps approach, which validation was simultaneously a goal of the present work. The first step consisted in the comparison of the products of a lower-cycle PCR amplification (25 cycles) of single strand cDNA libraries of both epigenomes, while a typical PCR amplification (35 cycles) was used as control for PCR efficiency, the second step consisted in conventional RT-qPCR analysis. The rational of such approach is the first step of analysis to be used as preliminary selection before confirmation by much more accurate RT-qPCR analysis. A previous, and easy to perform, pre-selection can speed up and increase the efficiency of all procedure when large amounts of expressed sequences require further RT-qPCR confirmation.

The next generation sequencing (Ion Torrent) of the cDNA-SSH libraries, resulted, respectively in 1337 contigs amplified from cv. Onward and 1282 contigs amplified from the “rogue” line JI2723. Among these contigs, 67 from the cv. Onward library and 60 from the “rogue” line library, exhibiting a redundancy equal or over 500 sequences in one of the epigenomes and being almost not present (less than 10 sequences) in the second epigenome, were selected for further RT-qPCR analysis (Annex II and Annex III).

In this work was performed the expression analysis of 24 of these sequences (Table 5 and 6).

Forty-eight primers were designed for amplification of short fragments (95 to 120 bp) within the analysed contigs and their optimal annealing temperatures were previously established (Table 7).

Table 5 Selected expressed sequences more abundant in the "rogue" line JI2723

Sequence name	Pea Contig	Onward vs Rogue	Rogue vs Onward
SSHR8	ID|Pisum_sativum_v2_Contig4568	0	1304
SSHR11	ID|Pisum_sativum_v2_Contig4871	0	1078
SSHR12	ID|Pisum_sativum_v2_Contig7001	4	1528
SSHR13	ID289409|p.sativum_wa1_contig24155	5	1157
SSRH14	ID|Pisum_sativum_v2_Contig4379	0	1039
SSHR15	ID|Pisum_sativum_v2_Contig8616	0	866
SSHR16	ID|Pisum_sativum_v2_Contig7306	3	889
SSHR17	ID|Pisum_sativum_v2_Contig5993	0	829
SSHR18	ID|Pisum_sativum_v2_Contig4801	0	807
SSHR19	ID|Pisum_sativum_v2_Contig5242	0	785
SSHR20	ID287628|p.sativum_wa1_contig23351	0	775

Table 6 Selected expressed sequences more abundant in cv, Onward

Sequence name	Pea Contig	Onward vs Rogue	Rogue vs Onward
SSHO2	ID281115|p.sativum_wa1_contig19650	1666	1
SSHO3	ID|Pisum_sativum_v2_Contig4380	1627	0
SSHO5	ID|Pisum_sativum_v2_Contig4909	1624	0
SSHO6	ID|Pisum_sativum_v2_Contig5891	1427	1
SSHO7	ID|Pisum_sativum_v2_Contig1549	1323	2
SSHO8	ID|Pisum_sativum_v2_Contig4284	1135	0
SSHO9	ID|Pisum_sativum_v2_Contig5916	1137	0
SSHO10	ID61637|Pisum_sativum_v1_Contig2226	1021	0
SSHO11	ID|Pisum_sativum_v2_Contig2333	1579	1
SSHO12	ID266692|p.sativum_wa1_contig30745	966	0
SSHO13	ID|Pisum_sativum_v2_Contig7337	1041	1
SSHO14	ID286784|p.sativum_wa1_contig06779	959	0
SSHO15	ID291839|p.sativum_wa1_contig18536	869	0

Table 7 Forward and reverse primers for amplification of pea conting fragments of interest.

Sequence	Primer e	Sequence	Fragment legth (bp)
SSHR8	R_Seq 8 Fw	TCTCCTTCATGGAGGTC	112
	R_Seq 8 Rv	AACACGTCAAGGACTCT	
SSHR11	R_Seq 11 Fw	TGACAACTTGCCTATGG	103
	R_Seq 11 Rv	ACTGATAAGGGCATCTC	
SSHR12	R_Seq 12 Fw	GAGTGGGACAGATTCAG	101
	R_Seq 12 Rv	TCAGCATCAATGTGACC	
SSHR13	R_Seq 13 Fw	TCATGCGGAGGACTATC	108
	R_Seq 13 Rv	CACCTTCCAAGCAAGG	
SSHR14	R_Seq 14 Fw	TCCACAGCAATTCTGTG	107
	R_Seq 14 Rv	AAGACATTCTCTGGCAAC	
SSHR15	R_Seq 15 Fw	AGACACAACTTGGATCC	117
	R_Seq 15 Rv	AATCGGTTGATCCTCAG	
SSHR16	R_Seq 16 Fw	TCCTCTAACTCTTCAAGCA	95
	R_Seq 16 Rv	TATGACTGTGGAAATGGAAG	
SSHR17	R_Seq 17 Fw	CTGCTGTTGATGATATTG	108
	R_Seq 17 Rv	TTAGCCTTAGAAGAAGC	
SSHR18	R_Seq 18 Fw	ACAACAGACGGTCATTG	106
	R_Seq 18 Rv	AATCGCTTCGGAAACTG	
SSHR19	R_Seq 19 Fw	TCTGCCATCGAGATATCA	105
	R_Seq 19 Rv	GTTCGCCTTTAACCAAG	
SSHR20	R_Seq 20 Fw	AATGATAGACATGGCAGATG	107
	R_Seq 20 Rv	AACAACCTGGCTTTGAG	
SSHO2	O_Seq 2 Fw	ATCTGCATCTGATTGTG	114
	O_Seq 2 Rv	CTCTGAATTATCAACTACAGA	
SSHO3	O_Seq 3 Fw	ATGGAGCACCAAGATATG	112
	O_Seq 3 Rv	AGATACAGAGATCAACCTC	
SSHO5	O_Seq 5 Fw	CAGCAGTGATAGCCATAG	120
	O_Seq 5 Rv	TGATTGAGAAGGCAACAC	
SSHO6	O_Seq 6 Fw	TCATTCTCCAAGGTTGCTG	100
	O_Seq 6 Rv	GGATACCTATCACCTAGAAC	
SSHO7	O_Seq 7 Fw	TTCTTCAGGTGTGCAAC	103
	O_Seq 7 Rv	TCCTGGTTGTCGATACTT	
SSHO8	O_Seq 8 Fw	TGAATTGCACTCCATCTC	104
	O_Seq 8 Rv	ATCCACTTTCTCCACTAC	
SSHO9	O_Seq 9 FW	TCAGCTCCAATTCTCCA	113
	O_Seq 9 Rv	GCTTGCCAAATGGATC	
SSHO10	O_Seq 10 Fw	AGTTCCTCGTAATCAGTGT	112
	O_Seq 10 Rv	TTCTTGCATCTAGAGCTC	

Table 7 Forward and reverse primers for amplification of pea conting fragments of interest. (cont.)

Sequence	Primer	Sequence	Fragment legth (bp)
SSHO11	O_Seq 11 Fw	ACATCTTCAATAGTTCCAAC	99
	O_Seq 11 Rv	ATACACCACTGTTTATGTTG	
SSHO12	O_Seq 12 Fw	TTGGTTGAACAAGCTTC	97
	O_Seq 12 Rv	GTCTCAACAACCAGATC	
SSHO13	O_Seq 13 Fw	TAAGGTTGACCGTGTG	118
	O_Seq 13 Rv	TGGCTCCTGCATAATG	
SSHO14	O_Seq 14 Fw	TTCTGGATTGTTTGAGGA	100
	O_Seq 14 Rv	TAACCAACTGAGCAACT	
SSHO15	O_Seq 15 Fw	ATTCGAGAAGGTATAGCATG	120
	O_Seq 15 Rv	TAGTAGGCATGGTCAGA	

Five out of the 24-SSH selected sequences have not showed visible and analysable PCR products after 25 cycles of PCR amplification (Table 8). In the remaining products of 25 cycles of PCR amplification 11 sequences have not showed differences for cDNA of cv. Onward and Onward "rogue" and the other 8 sequences have showed differences between the amplification products obtained from cDNA of cv. Onward and Onward "rogue" (Fig. 7 and Fig. 8, Table 8). The genes for Tubulin 2 and Polyubiquitin were used as controls in RT-qPCR and showed very balanced products of 25 and 35 cycles PCR amplification (Fig. 9).

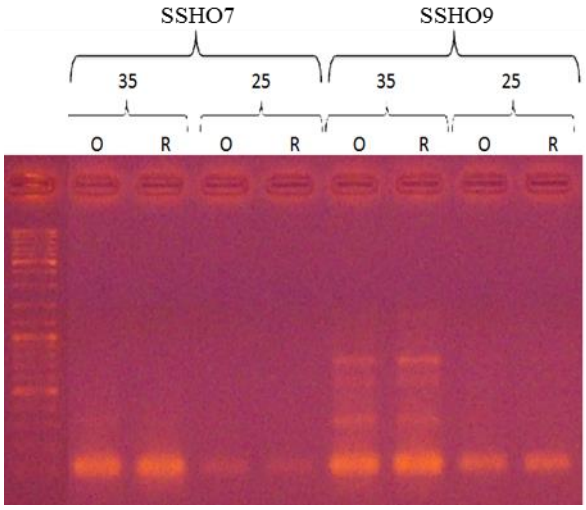

Figure 7 Preliminary PCR amplification for 35 and 25 cycles. No significant differences are noticeable between sampes (O and R) either amplified for 35 or 25 cycles. (O) - cv. Onward; (R) "Rogue" line.

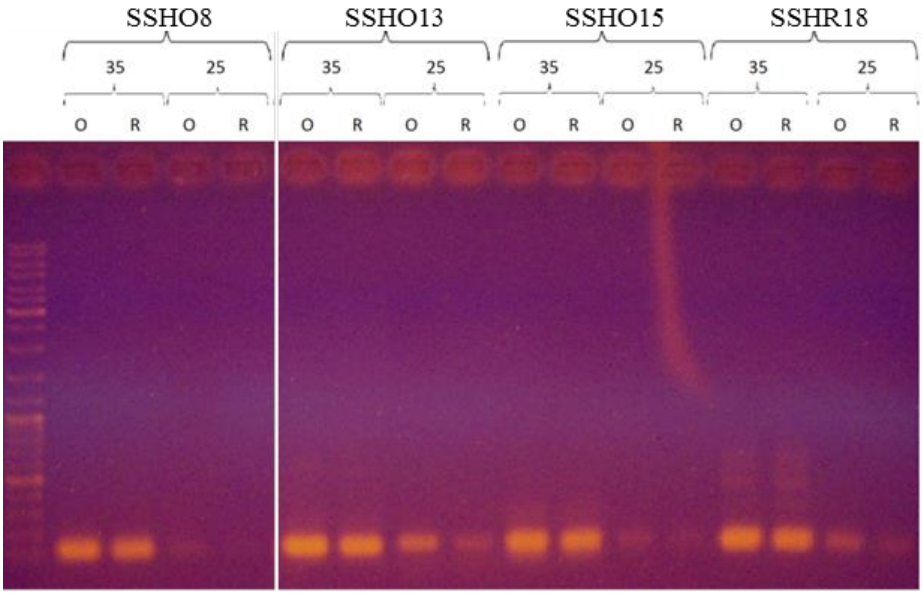

Figure 8 Some sequences exhibited differences in the products of 25 cycles PCR amplification. The differential expression of these sequences was later confirmed by RT-qPCR analysis.

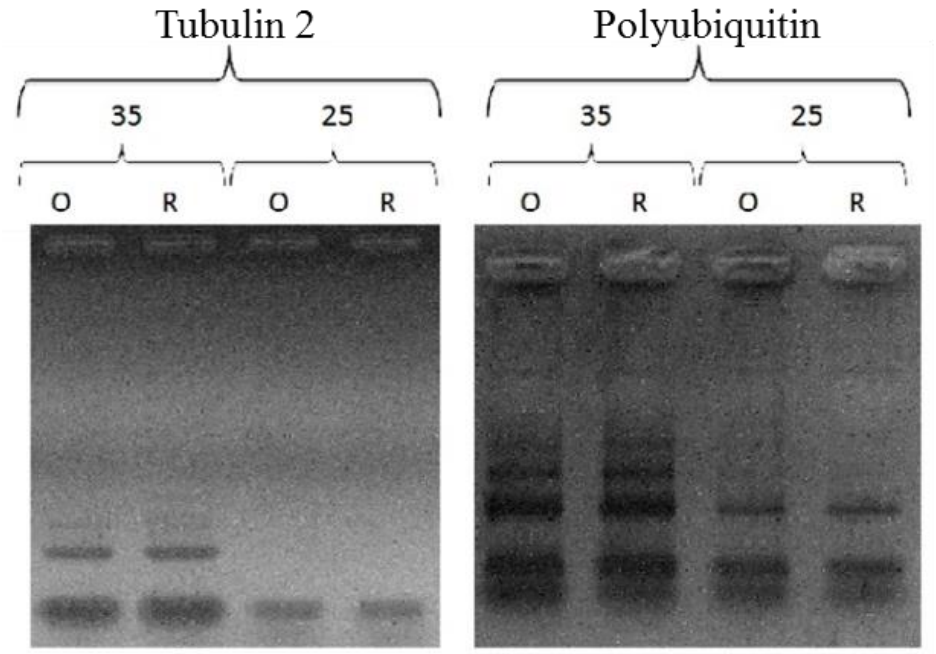

Figure 9 The Tubulin 2 and polyubiquitin genes used as control in RT-qPCR analyses showed similar results in cv. Onward (O) and Onward “rogue” line (R) after 35 or 25 cycles of PCR amplification.

The comparison between the results of RT-qPCR analysis and 25 cycle PCR amplification have showed that the risk of not selecting as priority for RT-qPCR sequences with highly significant differences in expression is apparently low.

All the sequences that exhibited differences in amplification after 25 cycles PCR have also showed significant differences in expression as assessed by RT-qPCR, whereas all the sequences that have not exhibited clear differences in the previous low-

Table 8 Results of 25 cycles PCR and RT-qPCR analysis.

Sequence	Differences after 25 cycles PCR	ΔCt	ΔΔCt	Validation 25 cycles PCR vs. RT-qPCR	Annealing Temperature (ºC)
SSHR8	No	-0,23	0,5	Confirmed	
SSHR11	-	-	-	-	
SSHR12	No	-1,43	-0,7	Confirmed	
SSHR13	No	-0,8	-0,067	Confirmed	
SSHR14	-	-	-	-	
SSHR15	No	-1,33	-0,6	Confirmed	
SSHR16	No	0,2	0,93	Confirmed	
SSHR17	No	-0,63	0,1	Confirmed	
SSHR18	**Yes**	**-3,1**	**-2,65**	**Confirmed**	**58**
SSHR19	**Yes**	**2,03**	**1,83**	**Confirmed**	**56**
SSHR20	No	1,25	1,7	Confirmed	
SSHO2	**-**	**2,8**	**2,6**	**-**	
SSHO3	No	-0,67	-0,87	Confirmed	
SSHO5	**Yes**	**-0,1**	**0,35**	**Negative**	**58**
SSHO6	**-**	**-2,5**	**-2,05**	**-**	
SSHO7	No	-1,5	-1,05	Confirmed	
SSHO8	**Yes**	**-2,75**	**-2,3**	**Confirmed**	**58**
SSHO9	No	-0,37	0,37	Confirmed	
SSHO10	**Yes**	**-2,25**	**-1,8**	**Confirmed**	**58**
SSHO11	-	-	-	-	
SSHO12	**Yes**	**-5,1**	**-4,65**	**Confirmed**	**58**
SSHO13	**Yes**	**-3,65**	**-3,2**	**Confirmed**	**58**
SSHO14	No	-0,8	-0,067	Confirmed	
SSHO15	**Yes**	**-1,55**	**-1,1**	**Confirmed**	**58**

-cycle PCR have not showed significant differences in RT-qPCR. In one case one sample apparently showed differences after 25 cycles PCR, which were not confirmed

by RT-qPCR. The pre-selection by low-cycle PCR can have a positive role when the RT-qPCR analysis is intended to be used for large amounts of sequences.

The analysis and discussion of the identified differences in gene expression between cv. Onward and Onward "rogue" line JI2723, in particular those showing highly significant differences ($\Delta\Delta Ct \geq 2,0$) is out of the scope of the present work and will be performed after additional results are obtained. Nevertheless, the first positive results on differential expression (which need further confirmation in at least two additional samples) are displayed in Table 9.

Table 9 Protein analyses the translation of fragments with differences in RT-qPCR

Sequence	ΔΔCt	Translated protein	Expect	Identify (%)
SSHR18	-2,65	Polyamine oxidase [Medicago truncatula] Sequence ID: ref|XP_003600621.1|Length: 492	0,0	95
SSHR19	1,83	Glycogen synthase kinase [Medicago truncatula] Sequence ID: ref|XP_003591238.1|Length: 411	0,0	97
SSHR20	1,7	Fructosamine kinase [Medicago truncatula] Sequence ID: gb|KEH18974.1|Length: 319	0,0	92
SSHO2	2,6	BEL1-related homeotic protein [Medicago truncatula] Sequence ID: gb|KEH41936.1 |Length: 649	0,0	90
SSHO6	-2,05	Glucose 6 phosphate/phosphate translocator-like protein [Medicago truncatula] Sequence ID: ref|XP_003594481.1|Length: 408	0,0	90
SSHO7	-1,05	Serine/threonine protein kinase ICK [Medicago truncatula] Sequence ID: ref|XP_003612616.1|Length: 449	0,0	90
SSHO8	-2,3	This contig does not translate in a viable protein	-	-
SSHO10	-1,8	Sucrose-phosphate synthase [Medicago truncatula] Sequence ID: ref|XP_003617418.1|Length: 1058	1e-106	88
SSHO12	-4,65	PREDICTED: SPX domain-containing protein 2-like [Glycine max] Sequence ID: ref|XP_003549761.1|Length: 295	2e-114	69
SSHO13	-3,2	Soluble inorganic pyrophosphatase [Medicago truncatula] Sequence ID: gb|KEH42358.1|Length: 248	2e-155	96
SSHO15	-1,1	DNA-directed RNA polymerase [Medicago truncatula] Sequence ID: ref|XP_003589372.1|Length: 1213	0,0	99

3.4- On the way to the expression analysis of genes, involved in DNA methylation and chromatin remodeling: *met1*, *ddm1*, *drm2* and *mop1*.

Recent studies developed in the laboratory of Genomics and Genetic Improvement, in the Universidade do Algarve using the method Methylation Sensitive - Amplified Fragment Length Polymorphisms (MS-AFLP) have identified a set of specific sequences that are differentially methylated in leaf DNA of cv. Onward and Onward "rogue" line JI2723. Some of the differential methylation patterns were even inherited through meiosis to pollen DNA (Santo and Leitão, unpublished results).

This finding raised interrogations regarding the expression of the genes involved in DNA-methylation in the emergence and maintenance of the "rogue" paramutation in pea (*Pisum sativum* L.). Tree genes: *ddm1; drm2* and *mop1*, were elected for further expression analysis.

However, no homologs of these three genes have been identified so far in this legume species, for which genomic data are still relatively meager. In order to retrieve in *Pisum* the sequence of these three genes, the genomic information on the legume model plant *Medicago truncatula* (http://www.jcvi.org/medicago) was used as reference for sequence analysis and primer design.

Amplifications of selected partial sequences of the *ddm1, drm2 and mop1* were performed using the primers designed based on the homolog *Medicago truncatula* sequences (Table 3). The amplification products were analyzed in agarose gels. When the amplification resulted in more than one amplification product the fragment of the right size was cut from the gel, purified and sent for Sanger sequencing. In the cases of a single product of right size, the fragment was ethanol precipitated directly in the PCR reaction mix and sent for sequencing.

The sequences and the putative protein products were blasted against the Gen Bank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

ddm1

The amplification of two sequences of *ddm1*(Fig. 10) was performed using the primers displayed in Table 10, which were also used as sequencing primers.

Table10 Primers for *ddm1* amplification and sequencing

Primer name	Primer sequense	bp	Annealing temperature (ºC)
DDM1exp_F6	AAGAACAATGTGAAGAACGA	617	55
DDM1exp_R5	TCAGCAAGAATCCCATTC		
DDM1_F10	TGCCTTTACTAACTGGTGG	989	55
DDM1 exp R4	GCATTGCTCAATTATCTC		

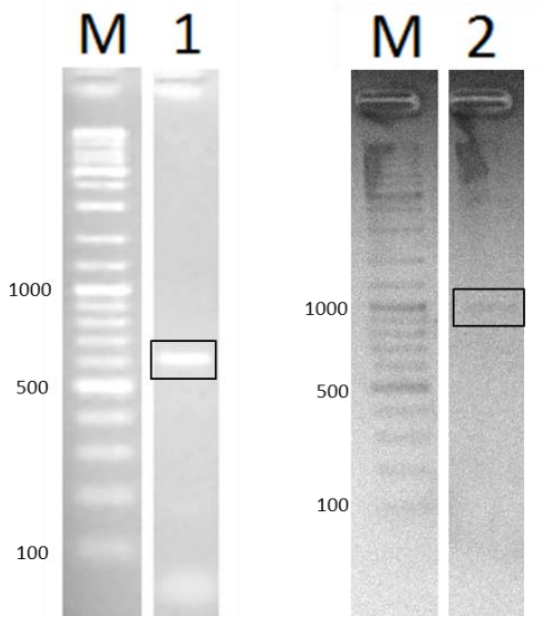

Figure 10 PCR amplification of two *ddm1*fragments in *Pisum sativum*. : 1) Product of amplification using the primers DDM1exp_F6 and DDM1exp_R5; 2) Product of amplification using the primers DDM1_F10 and DDM1 exp R4.

The nucleotide sequence of the PCR products and the respectively coded amino acid sequences were blasted against the GenBank database (www.ncbi.nlm.nih.gov) which confirmed that both sequences are similar to Swi2/Snf2-related chromatin remodeling ATPase (*Medicago truncatula*) (BOXES 1 and 2)

BOX1

***Psddm1* sequence** 1

```
TAAGAACAATGTGAAGAACGATGATCCTGCAGTAGAGTCACCAACTTCGGTCCTAGAAGAAGAGGAAATGGAGGTTAAG
TTGGAGGAAGAGGTGGTTGCAGATGATGGATCTTCTCTTGTACCGAAATTGATGGTAGAGGAGGAAGAGAAGTTGCTTA
AAGCTCTTGCTAAGGAAGAGGAGGAACAGTTTCAGGAGGCGCCAAATCTCAATGACTCGCAGTTTGATAAGTTGGATGA
GCTTTTAACGCAAACGAAACTGTACTCGGAGTTTCTTCTGGAGAAAATGGATGACATCACATTGACTGCGGGTGAACAA
GAGATTAAGGAAGAGGAAGAGACTCAGTTGGATACTAAACCTAAGGGTCGTGGAAGAAAAAGAAAGGCGGCTAAACAAT
GCAATACTGGGAAAGCCAAAAAGGCAGTTGAAGCTATGATAACAAGATCTAAAGAAACTGTGAAGACTGAAGATGTGAA
TTTGAGTGAGGAAGAAAGAACTGAGAAAGAGCAGAGGGAGTTGGTGCCTTTACTAACTGGTGGAAAATTGAAGTCTTAT
CAACTGAAGGGTGTAAAATGGTTAATCTCCTTGTGGCAAAATGGACTGAATGGGATTCTTGCTGAA
```

Swi2/Snf2-related chromatin remodeling ATPase[Medicago truncatula)
XP_003611980.1 Identity: 173/206(84%) Positives: 186/206(90%) Gaps: 0/206(0%)

```
Query  1    KNNVKNDDPAVESPTSVLEEEEMEVKLEEEVVADDGSSLVPKLMVEEEEKLLKALAKEEE  60
            KNNVKNDDP  ESPTSVLEEEE+EVK EEEV+ADDGSSLVPK M EEEEKLLK   KEEE
Sbjct  4    KNNVKNDDPPAESPTSVLEEEEVEVKSEEEVIADDGSSLVPKTMAEEEEKLLKVRVKEEE  63

Query  61   EQFQEAPNLNDSQFDKLDELLTQTKLYSEFLLEKMDDITLTAGEQEIKEEEETQLDTKPK  120
            E+ + APNLNDSQF+KLDELLTQTKLYSEFLLEKMDDIT+ AGEQE  +EEE++   K K
Sbjct  64   EKIEVAPNLNDSQFNKLDELLTQTKLYSEFLLEKMDDITMAAGEQEKPDEEESKPVAKKK  123

Query  121  GRGRKRKAAKQCNTGKAKKAVEAMITRSKETVKTEDVNLSEEERTEKEQRELVPLLTGGK  180
            GRG KRKAA QCNTGKAKKAVEAMITRSKE VKTEDV+L+EEERTEKEQREL+PLLTGGK
Sbjct  124  GRGSKRKAASQCNTGKAKKAVEAMITRSKENVKTEDVDLTEEERTEKEQRELMPLLTGGK  183

Query  181  LKSYQLKGVKWLISLWQNGLNGILAE  206
            LKSYQLKGVKWLISLWQNGLNGILA+
Sbjct  184  LKSYQLKGVKWLISLWQNGLNGILAD  209
```

drm2

Using the primers in Table 11 a fragment was amplified from cv. Onward (Fig. 11) which nucleotide sequence and putative protein product showed the highest similarity with the *drm1/drm2* genes of two other legume species *Cicer arietinum* and *Medicago trucatula* (BOX3).

Table 11 Primers for drm2 amplification and sequencing.

Primer name	Primer sequense	bp	Annealing temperature (ºC)
DRM2exp_F9	TGGTTGATACAATTGGAGAG	600	55
DRM2exp_R6	GTGAGGAGTTAGGACCT		

BOX 2

***Psddm1* sequence** 2

```
AAATGGTTAATTTCCTTGTGGCAAAATGGACTGAATGGGATTCTTGCTGATCAAATGGGTCTTGGGAAGACAATCCAGAC
AATTGGGCTTCTTTTTCATTTTAAATCAAAAGGATTGGATGGGCCATATATGATAATTGCTCCACTATCAACCCTATCCA
ACTGGATGAATGAGATATTTAGGTTTGCACCATCACTCCCTGCTGTTATCTACCACGGTAATAAAGATGAGAGAAATGAG
ATCAGAAGGAAACATATGCCTAGCACAATTGGTCCAAAATTTCCCATAGTAATAACTTcTTATGAGATTGCAATGAATGA
TGCTAAGAAATTTTTCCGGGCATACCAATGGAAATATCTTGTTGTTGATGAGGGTCACAGGcTAAAAAATTCACAATGCA
AATTAGTGACCATGTTGAAATTCATCAGAGTTGAAAATAAGCTTCTTTTGACTGGGACACCGCTCCAGAATAACTTAGCA
GAGCTGTGGTCATTGCTGAACTTCATCTTACCTGATATATTcTCATCTCTTGAAGAATTTGAGTCATGGTTTAATCTGTC
AGGAAAGTGTGCTTCTGGAGCAaCAATGGAAGAAATGGAAGAGAAAAGAAGAAACCAGGTAGTGGCCAAGCTTCATGCAA
TTCTCAGACCATTTCTTTTGCGCCGAATGAAGTCTGATGTTGAGCTATCATTGCCCCGGAAAAAAGAGATCATTATTTAT
GCTAACATGACTGAGCATCAGAAGAACTTGCAGGATCATcTAGTTAATGCGaCATTCGAGAAACATTTGGACAGGAAACT
AACAATTGGGCGTGCTGCGGCGAGTATTAATAACTTGGTAATTCAACTTAGGAAAGTCTGTAACCATCCCGACCTCTTAG
AATCACCCTATGATGGTTCATATTTTTATCCCTCCTTTGAATG
```

Swi2/Snf2-related chromatin remodeling ATPase [Medicago truncatula]
XP_003611980.1
Identities: 262/304(86%) Positives: 280/304(92%) Gaps: 0/304(0%)

```
Query  1    KWLISLWQNGLNGILADQMGLGKTIQTIGLLFHFKSKGLDGPYMIIAPLSTLSNWMNEIF  60
            KWLISLWQNGLNGILADQMGLGKTIQTIG L H KSKGLDGPYMIIAPLSTLSNWMNEI
Sbjct  193  KWLISLWQNGLNGILADQMGLGKTIQTIGFLSHLKSKGLDGPYMIIAPLSTLSNWMNEIN  252

Query  61   RFAPSLPAVIYHGNKDERNEIRRKHMPSTIGPKFPIVITSYEIAMNDAKKFFRAYQWKYL  120
            RF P+LPAVIYHGNK +R+EIRRKHMP T+GPKFP+VITSYEIAMNDAKK  R+Y WKYL
Sbjct  253  RFTPTLPAVIYHGNKHQRDEIRRKHMPRTVGPKFPLVITSYEIAMNDAKKCLRSYSWKYL  312

Query  121  VVDEGHRLKNSQCKLVTMLKFIRVENKLLLTGTPLQNNLAELWSLLNFILPDIFSSLEEF  180
             VDEGHRLKN+ CKLV MLK+I VENKLLLTGTPLQNNLAELWSLL+FILPDIFSSLEEF
Sbjct  313  AVDEGHRLKNANCKLVRMLKYISVENKLLLTGTPLQNNLAELWSLLHFILPDIFSSLEEF  372

Query  181  ESWFNLSGKCASGATMEEMEEKRRNQVVAKLHAILRPFLLRRMKSDVELSLPRKKEIIIY  240
            ESWFNLSGKC +GATMEE+EEKRR QVVAKLH+ILRPFLLRRMKSDVEL LPRKKEIIIY
Sbjct  373  ESWFNLSGKCTTGATMEELEEKRRTQVVAKLHSILRPFLLRRMKSDVELMLPRKKEIIIY  432

Query  241  ANMTEHQKNLQDHLVNATFEKHLDRKLTIGRAAASINNLVIQLRKVCNHPDLLESPYDGS  300
            ANMTEHQKNLQDHL+N T  K+LD+K +IGRA  S+NNLVIQLRKVCNHPDLLES +DGS
Sbjct  433  ANMTEHQKNLQDHLINETLGKYLDKKRSIGRAPTSLNNLVIQLRKVCNHPDLLESVFDGS  492

Query  301  YFYP  304
            YFYP
```

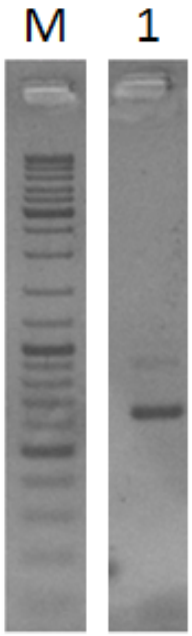

Figure 11 PCR amplification of a cDNA fragment of *Pisum sativum* using the primers DRM2exp_F9 and DRM2exp_R6.

BOX 3
***Psdrm2* sequence 1**

```
TTGGTTGATACAATTGGAGAGGAAGTTCACACGTCGTCCATTTTCGTGgAGCCCGAAGATTCGTCCTTTGCGAAATTGGG
AGACAAAATGAGTGATGATTCTGGTCTAGGGAGTGGAGGTTATGATTGGAATACTGAAGATGAGCTTGAGATTGAAAGCT
TTCATTCTTCCTGTACAACTGTTCCCTATGGACAGACTTCTGGGTCTCGGTCTCTAGAGGAAAACTCATTTGCAGCAGGT
CCATCTAATACCAAGGTGTTTGACTCTCTCATTAATATGGGATTTCATCCTGAAATGGTTGCCAAAGTAATTCAGGAATA
TGGTGAGGAAAATGAACATAAACTAGTTGAAGAGCTTCTCACATATCAAGAGCTAGAAAGGTCTTCTCAGCAGCAACAGC
AAGTTGAACCAGATCCCACCTCTTCAGAGTATGCAGCGAGCTCCTGGGATGATTCATCAGACAACGATGATTCATCGGAT
GAAGAAATACCAAAATCCCTTTCTAAGAATGATAATACATTACTATCCCTGGTAAAAATGGGATTCAATGAGGAGGAGGC
TTTAATGGCGTTAGAAAGATTAGGTCCTAACTCCTCA
```

DNA (cytosine-5)-methyltransferase DRM1/2 [Medicago truncatula] AES94878.2
Identities: 119/170(70%) Positives: 138/170(81%) Gaps: 1/170(0%)

```
Query  30   MSDDSGLGSGGYDWNTEDELEIESFHSSCTTVPYGQTSGSRSLEENSFAAGPSNTKVFDS  89
            M DDS L S  +DWNT+DELEIESF+S  +TVP  QT  + S+E +SFA GPSNTKV D
Sbjct  1    MGDDSSLESDNFDWNTDDELEIESFNSLSSTVPSRQTITAASVEASSFA-GPSNTKVLDH  59

Query  90   LINMGFHPEMVAKVIQEYGEENEHKLVEELLTYQELERSSQQQQQVEPDPTSSEYAASSW  149
             I+MGF  E+V+KVIQEYGEE+E KL+EE+LTY  LE SSQQ QQVEPDPTSSEYA SSW
Sbjct  60   FISMGFPGEVVSKVIQEYGEEDEDKLLEEILTYSALESSSQQHQQVEPDPTSSEYAGSSW  119

Query  150  DDSSDNDDSSDEEIPKSLSKNDNTLLSLVKMGFNEEEALMALERLGPNSS  199
            DD SD D  SDEE+PKS+S+ND+TLLSLV MGF EEEALMA+ERLG +SS
Sbjct  120  DDLSDGDSFSDEEMPKSVSRNDDTLLSLVNMGFKEEEALMAIERLGLDSS  169
```

mop1

A piece of the *mop1* sequence in Psium sativum was amplified using the primers displayed in Table 12. The amplification product had the expected size (Fig. 12) and, after precipitation, was sent directly for sequencing. The blastn and blastp against the GenBank database (www.ncbi.nlm.nih.gov) confirmed that the amplified sequence is related to an RNA-dependent RNA polymerase 2-like sequence in chickpeas (*Cicer arietinum*) similar to the *mop1* gene involved in the paramutation of the locus *b1* in *Zea mays* (BOX 4).

Table 12 Primers for amplification and sequencing of mop1 homolog sequence in *Pisum sativum*.

Primer name	Primer sequense	bp	Annealing temperature (ºC)
MOP_FW1p	TGAAGAAGCATATGATCATCAAC	407	55
MOP_Rv3p	CAGACTTGATATGCAATAAAATGTC		

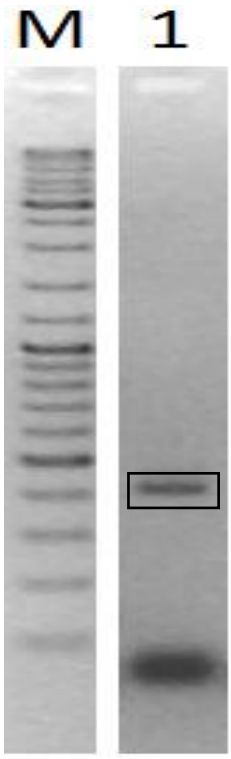

Figure 12 PCR amplification of a putative mop1fragment in Pisum sativum. Product of amplification using the primers MOP_FW1and MOP_Rv3p.

BOX 4

***Psmop1* sequence**:

```
TTGAAGAAGCATATGATCATCAACTTGAGGTTAATGGTTTTGAGGTCTTTCTTGAGACTGCCTCAAGTCACAGAGAAATGT
ATGCACAGAAGATGAGCTCTTTAATGAGCTTCTATGGAGCAGAGACCGAGGATGAAATGCTAACAGGTAACTTGCTAAAAC
GTGCTTCTTATTTGCAGCGCGATAACAGGAGATATGGAGATATGAAGGATCGAATTCTGATATCGGTGAAGGATCTTCAAC
ATGAAGCTAAAGGATGGTTTGAAAGTGATTGTCAGCCAGATGAATATCAACTTATGGCATCTGCATGGTATCATGTGACCT
ATCATCCCAAATATTACCACGAAAGCTCCACCTTTTTAAGCTTCCCATGGATCGTTGGTGACATTTTATTGCATATCAAGT
CTGA
```

PREDICTED: **RNA-dependent RNA polymerase 2-like [Cicer arietinum]** XP_004508850.1
Identities: 126/135(93%) Positives: 127/135(94%) Gaps: 0/135(0%)

```
Query  1     EEAYDHQLEVNGFEVFLETASSHREMYAQKMSSLMSFYGAETEDEMLTGNLLKRASYLQR  60
             EEAYDHQLEVNGFE FLETASSH+EMYAQKMSSLMSFY AETEDEMLTGNL  RASYLQR
Sbjct  981   EEAYDHQLEVNGFEAFLETASSHKEMYAQKMSSLMSFYDAETEDEMLTGNLQNRASYLQR  1040

Query  61    DNRRYGDMKDRILISVKDLQHEAKGWFESDCQPDEYQLMASAWYHVTYHPKYYHESSTFL  120
             DNRRYGDMKDRILISVKDLQ EAK WFESDCQP EYQLMASAWYHVTYHPKY HESSTFL
Sbjct  1041  DNRRYGDMKDRILISVKDLQREAKEWFESDCQPHEYQLMASAWYHVTYHPKYSHESSTFL  1100

Query  121   SFPWIVGDILLHIKS  135
             SFPWIVGDILLHIKS
Sbjct  1101  SFPWIVGDILLHIKS  1115
```

3.5 siRNA isolation

The isolation of siRNA from total RNA was performed using a first step of PEG and NaCl precipitation of the high molecular weight (HMW) RNA. The low molecular weight fraction showed to harbour RNAs of the expected siRNA length (19-24bp) (Figure 13) which were excised and purified from the polyacrylamide gel for adapter ligation.

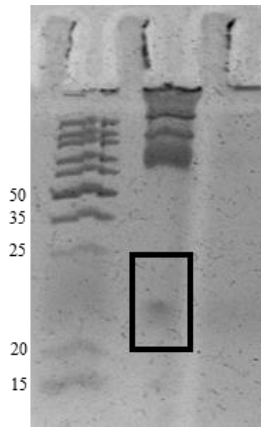

Figure 13 Separation in polyacrylamide/urea gel of LMW RNA. Notice the RNA signal with the expected size of siRNA .

After the ligation of the adapters, with 26 nucleotides each, the obtained cDNA was expected to be in the 71 to 76 bp range. However, the PCR amplification using primers complementary to the adapter sequences resulted in a strong band of approximately 50 bp, most probably resulting from the amplification of ligated adapters, and stronger signal in the 75 bp and150 bp size (Fig. 14). The fraction of these two gel regions were excised (Fig.15) and purified, after 6 cycles of PCR amplification, for a further analysis and utilization.

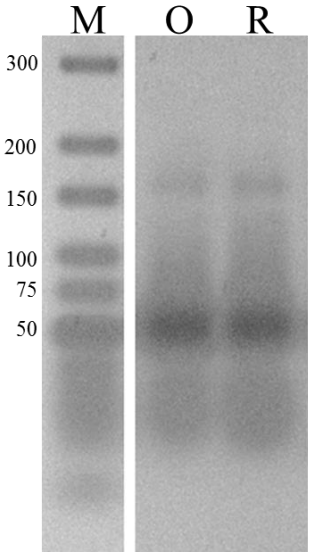

Figure 14 PCR amplification of small RNAs derived cDNA (O) cv. Onward; (R) “rogue” line JI2723; (M) Marker - Thermo scientific GeneRuler Ultra Low Range DNA Ladder.

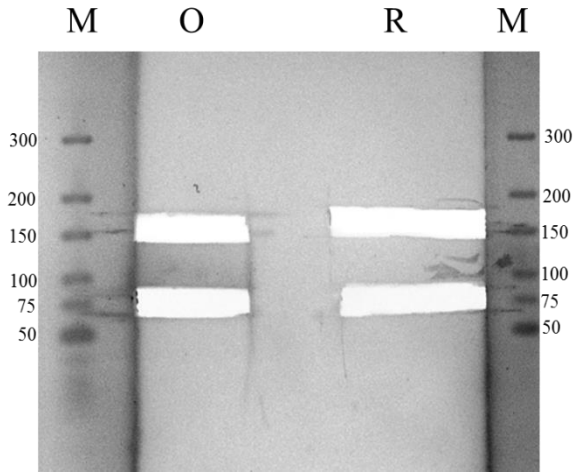

Figure 15 Excision of the 75 bp and 150 bp cDNAs after 6 cycles PCR amplification (O) cv. Onward; (R) “rogue” line JI2723; (M) Marker - Thermo scientific GeneRuler Ultra Low Range DNA Ladder

4- Discussion and future prospects

The study of “rogue” phenotype in pea was almost completely abandoned for approximately nine decades since the studies on the inheritance of this phenotype were carried out by Bateson and Pellew [21][22] and, immediately after, by Brotherton [29] in the early XX century. The study of this *Pisum* paramutation was later complemented with the observation of the lack of differences between the chromosomes of “rogue” and “non-rogue” plants [30], and that the reduced size of the leaves in “rogue” plants was caused by lower number of cells and not by differences in their size [31].

Recently, the research on this paramutation was resumed at the Laboratory of Genomics and Genetic Improvement, FCT, Universidade do Algarve, where was found that in a background of a similar level of genome wide methylation in leaf DNA, some specific genomic sequences exhibited altered methylation patterns in paramutated plants. In some cases the specific patterns were even inherited through meiosis to pollen DNA. Moreover, a non-rogue mutant line was induced from a Rogue pea cultivar, which evidenced the existence of specific genes which function is required for the Rogue paramutation process (Santo and Leitão, unpublished results).

The next generation sequencing of two SSH libraries, respectively from cv. Onward and paramutated Onward rogue line JI2723, resulted in over one thousand cDNA sequences showing significant differences in their redundancy, a consequence of differences in number of the respective mRNA molecules, between both epigenomes.

The present study aimed to identify differences in gene expression between the two epigenomes. The work was initiated with the multi-RAPD differential display analysis of cDNA libraries, however, in spite of the use of combinations of over 400 primers, no expression polymorphisms have been identified. Then, it was decided to search for differences in gene expression analysing and confirming by RT-qPCR the putative differential expression of sequences previously identified using a SSH approach.

Twenty-four sequences were selected for this study and 11 out of them showed to be differentially expressed. Eight of these last sequences exhibited a $\Delta\Delta Ct \geq 2$, assumed to be a highly significant value. These sequences may be associated with molecular mechanisms related with the paramutation. Nevertheless, they need to be confirmed using additional biological samples, which in these experiments consist on cDNA synthesized from the mRNA of three plants of each epigenome. During this

study a method was tested and validated for pre-selection of the sequences for further RT-qPCR assay, based on the comparative results of 25 cycles PCR.

As it was found that paramutated plants exhibited differences in DNA methylation the next logical step regarding this aspect of the paramutation was to assess the expression of genes related with DNA methylation and chromatin remodelling. The retrieving of expressed tags of the genes *ddm1*,*drm2* and *mop1* in *Pisum sativum* has created the conditions for the study of the expression of these genes in the two contrasting epigenomes.

The comparative analyses of siRNA libraries, still in construction for next generation (ion torrent) sequencing, is expected to provide some clues regarding the modulating mechanisms involved in the establishment of the Rogue paramutation.

Besides the study of the siRNAs, particularly aiming to identify different classes of these ncRNAs in both epigenomes, from the above results it can be concluded that the future research should focus on the screening of the sequences identified by SSH analyses, which initial study was very promising and eventually leading to the identification of metabolic pathways involved in the paramutation process.

6- References

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Annex I

Table A 1 Primers used in the Multi-RAPD differential display analysis.

Mix	Name	Sequence	Mix	Name	Sequence	Mix	Name	Sequence
1	OPAL-01	TGTGACGAGG	11	OPAM-03	CTTCCCTGTG	21	OPAO-04	AACAGGGCAG
	OPAL-02	ACCCTGTGGG		OPAM-04	GAGGGACCTC		OPAO-05	TGGAAGCACC
	OPAL-03	CCCACCCTTG		OPAM-05	GGGCTATGCC		OPAO-06	AGGCAGCCTG
2	OPAL-04	ACAACGGTCC	12	OPAM-06	CTCGGGATGT	22	OPAO-07	GATGCGACGG
	OPAK-05	GATGGCAGTC		OPAM-07	AACCGCGGCA		OPAO-08	ACTGGCTCTC
	OPAK-06	TCACGTCCCT		OPAM-08	ACCACGAGTG		OPAO-09	CCAGATGGGG
	OPAK-07	CTTGGGGGAC		OPAM-09	TGCCGGTTCA		OPAO-10	GACATCGTCC
3	OPAK-08	CCGAAGGGTG	13	OPAM-10	CAGACCGACC	23	OPAO-11	GGGGGCTTGA
	OPAK-09	AGGTCGGCGT		OPAM-11	AGATGCGCGG		OPAO-12	TCCCGGTCTC
	OPAK-10	CAAGCGTCAC		OPAM-12	TCTCACCGTC		OPAO-13	CCCACAGGTG
	OPAK-11	CAGTGTGCTC		OPAM-13	CACGGCACAA		OPAO-14	CTACTGGGGT
4	OPAK-12	AGTGTAGCCC	14	OPAM-14	TGGTTGCGGA	24	OPAO-15	GAAGGCTCCC
	OPAK-13	TCCCACGAGT		OPAM-15	GATGCGATGG		OPAO-16	CACAACGGGA
	OPAK-15	ACCTGCCGTT		OPAM-16	TGGCGGTTTG		OPAO-17	CCCATGTGTG
	OPAK-16	CTGCGTGCTC		OPAM-17	CCTAACGTCC		OPAO-19	GTTCTCGGAC
5	OPAK-17	CAGCGGTCAC	15	OPAM-18	ACGGGACTCT	25	OPAO-20	GGCTTGCCTG
	OPAK-18	ACCCGGAAAC		OPAM-19	CCAGGTCTTC		OPAK-01	TCTGCTACGG
	OPAK-19	TCGCAGCGAG		OPAM-20	ACCAACCAGG		OPAK-02	CCATCGGAGG
	OPAK-20	TGATGGCGTC		OPAN-01	ACTCCACGTC		OPAK-03	GGTCCTACCA
6	OPAL-01	TGTGACGAGG	16	OPAN-02	CACCGCAGTT	26	OPAK-04	AGGGTCGGTC
	OPAL-02	ACCCTGTGGG		-	-		OPAA-01	AGACGGCTCC
	OPAL-03	CCCACCCTTG		OPAN-04	GGCGTAAGTC		OPAA-03	TTAGCGCCCC
	OPAL-04	ACAACGGTCC		OPAN-05	GGGTGCAGTT		OPAA-04	AGGACTGCTC
7	OPAL-05	GACTGCGCCA	17	OPAN-06	GGGAACCCGT	27	OPAA-05	GGCTTTAGCC
	OPAL-06	AAGCGTCCTC		OPAN-07	TCGCTGCGGA		OPAA-06	GTGGGTGCCA
	OPAL-07	CCGTCCATCC		OPAN-08	AAGGCTGCTG		OPAA-07	CTACGCTCAC
	OPAL-08	GTCGCCCTCA		OPAN-09	GGGGGAGATG		OPAA-08	TCCGCAGTAG
8	OPAL-09	CAGCGAGTAG	18	OPAN-10	CTGTGTGCTC	28	OPAA-09	AGATGGGCAG
	OPAL-10	AAGGCCCCTG		OPAN-11	GTCCATGCAG		OPAA-10	TGGTCGGGTG
	OPAL-11	GTCACGTCCT		OPAN-12	AACGGCGGTC		OPAA-11	ACCCGACCTG
	OPAL-12	CCCAGGCTAC		OPAN-13	CTTCCAGGAC		OPAA-12	GGACCTCTTG
9	OPAL-13	GAATGGCACC	19	OPAN-14	AGCCGGGTAA	29	OPAA-13	GAGCGTCGCT
	OPAL-14	TCGCTCCGTT		OPAN-15	TGATGCCGCT		OPAA-14	AACGGGCCAA
	OPAL-15	AGGGGACACC		OPAN-16	GTGTCGAGTC		OPAA-15	ACGGAAGCCC
	OPAL-16	CTTTCGAGGG		OPAN-17	TCAGCACAGG		OPAA-16	GGAACCCACA
10	OPAL-17	CCGCAAGTGT	20	OPAN-18	TGTCCTGCGT	30	OPAA-17	GAGCCCGACT
	OPAL-18	GGAGTGGACT		OPAN-19	ACCACGCCTT		OPAA-18	TGGTCCAGCC
	OPAL-19	TCTGCCAGTG		OPAN-20	GAGTCCTCAC		OPAA-19	TGAGGCGTGT
	OPAL-20	AGGAGTCGGA		OPAO-01	AAGACGACGG		OPAA-20	TTGCCTTCGG
	OPAM-02	ACTTGACGGG		OPAO-02	AATCCGCTGG		OPAB-01	CCGTCGGTAG

Table A 1 Primers used in the Multi-RAPD differential display analysis. (cont.)

Mix	Name	Sequence	Mix	Name	Sequence	Mix	Name	Sequence
	OPAB-02	GGAAACCCCT		OPAD-03	TCTCGCCTAC		OP Z-06	GTGCCGTTCA
31	OPAB-03	TGGCGCACAC	41	OPAD-04	GTAGGCCTCA	51	OP Z-07	CCAGGAGGAC
	OPAB-05	CCCGAAGCGA		OPAD-06	AAGTGCACGG		OP Z-08	GGGTGGGTAA
	OPAB-07	GTAAACCGCC		OPAD-07	CCCTACTGGT		OP Z-09	CACCCCAGTC
	OPAB-06	GTGGCTTGGA		OPAD-08	GGCAGGCAAG		OP Z-10	CCGACAAACC
32	OPAB-08	GTTACGGACC	42	OPAD-09	TCGCTTCTCC	52	OP Z-11	CTCAGTCGCA
	OPAB-09	GGGCGACTAC		OPAD-10	AAGAGGCCAG		OP Z-12	TCAACGGGAC
	OPAB-10	TTCCCTCCCA		OPAD-11	CAATCGGGTC		OP Z-13	GACTAAGCCC
	OPAB-11	GTGCGCAATG		OPAD-12	AAGAGGGCGT		OP Z-14	TCGGAGGTTC
33	OPAB-12	CCTGTACCGA	43	OPAD-13	GGTTCCTCTG	53	OP Z-15	CAGGGCTTTC
	OPAB-13	CCTACCGTGG		OPAD-14	GAACGAGGGT		OP Z-16	TCCCCATCAC
	OPAB-14	AAGTGCGACC		OPAD-15	TTTGCCCCGT		OP Z-17	CCTTCCCACT
	OPAB-15	CCTCCTTCTC		OPAD-16	AACGGGCGTC		OP S-01	CTACTGCGCT
34	OPAB-16	CCCGGATGGT	44	OPAD-17	GGCAAACCCT	54	OP S-02	CCTCTGACTG
	OPAB-17	TCGCATCCAG		OPAD-18	ACGAGAGGCA		OP S-03	CAGAGGTCCC
	OPAB-18	CTGGCGTGTC		OPAD-19	CTTGGCACGA		OP S-04	CACCCCCTTG
	OPAB-19	ACACCGATGG		OPAD-20	TCTTCGGAGG		OP S-05	TTTGGGGCCT
35	OPAB-20	CTTCTCGGAC	45	OPAE-02	TCGTTCACCC	55	OP S-06	GATACCTCGG
	OPAC-01	TCCCAGCAGA		OPAE-03	CATAGAGCGG		OP S-07	TCCGATGCTG
	OPAC-02	GTCGTCGTCT		OPAE-04	CCAGCACTTC		OP S-08	TTCAGGGTGG
	OPAC-03	CACTGGCCCA		OPAE-05	CCTGTCAGTG		OP S-09	TCCTGGTCCC
36	OPAC-04	ACGGGACCTG	46	OPAE-06	GGGGAAGACA	56	OP S-10	ACCGTTCCAG
	OPAC-05	GTTAGTGCGG		OPAE-07	GTGTCAGTGG		OP S-12	CTGGGTGAGT
	OPAC-06	CCAGAACGGA		OPAE-08	CTGGCTCAGA		OP S-14	AAAGGGGTCC
	OPAC-07	GTGGCCGATG		OPAE-09	TGCCACGAGG		OP S-13	GTCGTTCCTG
37	OPAC-08	TTTGGGTGCC	47	OPAE-10	CTGAAGCGCA	57	OP Z-19	GTGCGAGCAA
	OPAC-09	AGAGCGTACC		OPAE-11	AAGACCGGGA		OP S-15	CAGTTCACGG
	OPAC-10	AGCAGCGAGG		OPAE-12	CCGAGCAATC		OP S-16	AGGGGGTTCC
	OPAC-11	CCTGGGTCAG		OPAE-13	TGTGGACTGG		OP S-18	CTGGCGAACT
38	OPAC-12	GGCGAGTGTG	48	OPAE-14	GAGAGGCTCC	58	OP S-19	GAGTCAGCAG
	OPAC-13	GACCCGATTG		OPAE-15	TGCCTGGACC		OP S-20	TCTGGACGGA
	OPAC-14	GTCGGTTGTC		OPAE-16	TCCGTGCTGA		OP T-01	GGGCCACTCA
	OPAC-15	TGCCGTGAGA		OPAE-17	GGCAGGTTCA		OP T-02	GGAGAGACTC
39	OPAC-16	CCTCCTACGG	49	OPAE-18	CTGGTGCTGA	59	OP T-03	TCCACTCCTG
	OPAC-17	CCTGGAGCTT		OPAE-19	GACAGTCCCT		OP T-04	CACAGAGGGA
	OPAC-18	TTGGGGGAGA		OPAE-20	TTGACCCCAG		OP T-05	GGGTTTGGCA
	OPAC-19	AGTCCGCCTG		OP Z-02	CCTACGGGGA		OP T-06	CAAGGGCAGA
40	OPAC-20	ACGGAAGTGG	50	OP Z-03	CAGCACCGCA	60	OP T-07	GGCAGGCTGT
	OPAD-01	CAAAGGGCGG		OP Z-04	AGGCTGTGCT		OP T-09	CACCCCTGAG
	OPAD-02	CTGAACCGCT		OP Z-05	TCCCATGCTG		OP U-07	CCTGCTCATC

Table A 1 Primers used in the Multi-RAPD differential display analysis. (cont.)

Mix	Name	Sequence	Mix	Name	Sequence	Mix	Name	Sequence
	OP T-10	CCTTCGGAAG		OP X-03	TGGCGCAGTG		OPAK-18	ACCCGGAAAC
61	OP T-11	TTCCCCGCGA	71	OP X-05	CCTTTCCCTC	81	OPAL-02	ACCCTGTGGG
	OP T-12	GGGTGTGTAG		OP X-07	GAGCGAGGCT		OPAL-06	AAGCGTCCTC
	OP T-13	AGGACTGCCA		OP G-06	GTGCCTAACC		OPAL-10	AAGGCCCCTG
	OP T-14	AATGCCGCAG		OP X-08	CAGGGGTGGA		OPAL-14	TCGCTCCGTT
62	OP T-15	GGATGCCACT	72	OP X-09	GGTCTGGTTG	82	OPAL-18	GGAGTGGACT
	OP T-16	GGTGAACGCT		OP X-10	CCCTAGACTG		OPAM-03	CTTCCCTGTG
	OP T-17	CCAACGTCGT		OP X-12	TCGCCAGCCA		OPAM-07	AACCGCGGCA
	OP T-18	GATGCCAGAC		OP X-14	ACAGGTGCTG		OPAM-11	AGATGCGCGG
63	OP T-19	GTCCGTATGG	73	OP X-15	CAGACAAGCC	83	OPAM-15	GATGCGATGG
	OP T-20	GACCAATGCC		OP X-17	GACACGGACC		OPAM-19	CCAGGTCTTC
	OP U-03	CTATGCCGAC		OP X-18	GACTAGGTGG		OPAN-04	GGCGTAAGTC
	OP U-02	CTGAGGTCTC		OP X-19	TGGCAAGGCA		OPAN-07	TCGCTGCGGA
64	OP U-04	ACCTTCGGAC	74	OP X-20	CCCAGCTAGA	84	OPAN-11	GTCCATGCAG
	OP U-05	TTGGCGGCCT		OP Y-01	GTGGCATCTC		OPAN-15	TGATGCCGCT
	OP U-06	ACCTTTGCGG		OP Y-02	CATCGCCGCA		OPAN-19	ACCACGCCTT
	OP U-11	AGACCCAGAG		OP Y-03	ACAGCCTGCT		OPAO-04	AACAGGGCAG
65	OP U-12	TCACCAGCCA	75	OP Y-04	GGCTGCAATG	85	OPAO-08	ACTGGCTCTC
	OP U-13	GGCTGGTTCC		OP Y-05	GGCTGCGACA		OPAO-12	TCCCGGTCTC
	OP U-14	TGGGTCCCTC		OP Y-06	AAGGCTCACC		OPAO-16	CACAACGGGA
	OP V-01	TGACGCATGG		OP Y-07	AGAGCCGTCA		OPAK-01	TCTGCTACGG
66	OP V-02	AGTCACTCCC	76	OP Y-08	AGGCAGAGCA	86	OPAA-01	AGACGGCTCC
	OP V-03	CTCCCTGCAA		OP Y-09	AGCAGCGCAC		OPAA-06	GTGGGTGCCA
	OP V-04	CCCCTCACGA		OP Y-10	CAAACGTGGG		OPAA-10	TGGTCGGGTG
	OP V-05	TCCGAGAGGG		OP Y-11	AGACGATGGG		OPAA-14	AACGGGCCAA
67	OP V-06	ACGCCCAGGT	77	OP Y-12	AAGCCTGCGA	87	OPAA-18	TGGTCCAGCC
	OP V-08	GGACGGCGTT		OP Y-13	GGGTCTCGGT		OPAB-02	GGAAACCCCT
	OP V-09	TGTACCCGTC		OP Y-14	GGTCGATCTG		OPAB-06	GTGGCTTGGA
	OP V-10	GGACCTGCTG		OP Y-15	AGTCGCCCTT		OPAB-11	GTGCGCAATG
68	OP V-11	CTCGACAGAG	78	OP Y-17	GACGTGGTGA	88	OPAB-15	CCTCCTTCTC
	OP V-12	ACCCCCCACT		OP Y-18	GTGGAGTCAG		OPAB-19	ACACCGATGG
	OP V-13	ACCCCCTGAA		OP Y-19	TGAGGGTCCC		OPAC-03	CACTGGCCCA
	OP V-14	AGATCCCGCC		OP G-02	GGCACTGAGG		OPAC-07	GTGGCCGATG
69	OP V-15	CAGTGCCGGT	79	OP G-03	GAGCCCTCCA	89	OPAC-11	CCTGGGTCAG
	OP V-16	ACACCCCACA		OP G-04	AGCGTGTCTG		OPAC-15	TGCCGTGAGA
	OP V-17	ACCGGCTTGT		OP G-08	TCACGTCCAC		OPAC-19	AGTCCGCCTG
	OP V-18	TGGTGGCGTT		OPAL-01	TGTGACGAGG		OPAD-03	TCTCGCCTAC
70	OP V-19	GGGTGTGCAG	80	OPAK-05	GATGGCAGTC	90	OPAD-08	GGCAGGCAAG
	OP V-20	CAGCATGGTC		OPAK-09	AGGTCGGCGT		OPAD-12	AAGAGGGCGT
	OP G-07	GAACCTGCGG		OPAK-13	TCCCACGAGT		OPAD-16	AACGGGCGTC

Table A 1 Primers used in the Multi-RAPD differential display analysis. (cont.)

Mix	Name	Sequence	Mix	Name	Sequence	Mix	Name	Sequence
91	OPAD-20	TCTTCGGAGG	98	OP X-14	ACAGGTGCTG	105	OPAO-05	TGGAAGCACC
	OPAE-05	CCTGTCAGTG		OP X-19	TGGCAAGGCA		OPAO-09	CCAGATGGGG
	OPAE-09	TGCCACGAGG		OP Y-03	ACAGCCTGCT		OPAO-13	CCCACAGGTG
	OPAE-13	TGTGGACTGG		OP Y-07	AGAGCCGTCA		OPAO-17	CCCATGTGTG
	OPAE-17	GGCAGGTTCA		OP Y-11	AGACGATGGG		OPAK-02	CCATCGGAGG
92	OP Z-02	CCTACGGGGA	99	OP Y-15	AGTCGCCCTT	106	OPAA-03	TTAGCGCCCC
	OP Z-06	GTGCCGTTCA		OP G-02	GGCACTGAGG		OPAA-07	CTACGCTCAC
	OP Z-10	CCGACAAACC		OP G-08	TCACGTCCAC		OPAA-11	ACCCGACCTG
	OP Z-14	TCGGAGGTTC		OPAL-02	ACCCTGTGGG		OPAA-15	ACGGAAGCCC
93	OP S-01	CTACTGCGCT	100	OPAK-06	TCACGTCCCT	107	OPAA-19	TGAGGCGTGT
	OP S-05	TTTGGGGCCT		OPAK-10	CAAGCGTCAC		OPAB-03	TGGCGCACAC
	OP S-09	TCCTGGTCCC		OPAK-15	ACCTGCCGTT		OPAB-08	GTTACGGACC
	OP S-13	GTCGTTCCTG		OPAK-19	TCGCAGCGAG		OPAB-12	CCTGTACCGA
94	OP S-18	CTGGCGAACT	101	OPAL-03	CCCACCCTTG	108	OPAB-16	CCCGGATGGT
	OP T-02	GGAGAGACTC		OPAL-07	CCGTCCATCC		OPAB-20	CTTCTCGGAC
	OP T-06	CAAGGGCAGA		OPAL-11	GTCACGTCCT		OPAC-04	ACGGGACCTG
	OP T-10	CCTTCGGAAG		OPAL-15	AGGGGACACC		OPAC-08	TTTGGGTGCC
95	OP T-14	AATGCCGCAG	102	OPAL-19	TCTGCCAGTG	109	OPAC-12	GGCGAGTGTG
	OP T-18	GATGCCAGAC		OPAM-04	GAGGGACCTC		OPAC-16	CCTCCTACGG
	OP U-02	CTGAGGTCTC		OPAM-08	ACCACGAGTG		OPAC-20	ACGGAAGTGG
	OP U-11	AGACCCAGAG		OPAM-12	TCTCACCGTC			
96	OP V-01	TGACGCATGG	103	OPAM-16	TGGCGGTTTG			
	OP V-05	TCCGAGAGGG		OPAM-20	ACCAACCAGG			
	OP V-10	GGACCTGCTG		OPAN-05	GGGTGCAGTT			
	OP V-14	AGATCCCGCC		OPAN-08	AAGGCTGCTG			
97	OP V-18	TGGTGGCGTT	104	OPAN-12	AACGGCGGTC			
	OP X-03	TGGCGCAGTG		OPAN-16	GTGTCGAGTC			
	OP X-08	CAGGGGTGGA		OPAN-20	GAGTCCTCAC			

Annex II

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Rogue = 1304 Onward = 0

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ACGCTACGCTGCACTTGAACAAACTATTAACTCTCTCGACCGTCAGATTTCGAATCATCTTTCGTCGGAAGATTTCAT
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TCGATCACTCATGGAACTCGGAGGCGAGTCGTTTGACTTGACTCGGAGTAAGGAGAAGTCAACTCAGAACGGGAACTC
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CGCTGATGCTGTAGCCATTGGTAGTCGCACGCCGGAGCGGTTGTTTAGAGTCCTTGACGTGTTTGAGACATTGCGTGA
CCTAATTCCCGAGTTTGAAGCATTGTTCTCTGATCAGTATTGCTCGTTCCTTGTAAATGAAGCTATCACAATTTGGAA
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Rogue = 1078 Onward = 1

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CTTCCATTGCAGAAGATCAACAACAACAACAACAACAACAACAACACTTGCTGTTGTTTCTCTTTCCTTGTTGATTCTCTT
CTCCACTTCTAGTTTCGTTCGTGCATCTTCTTCTATCTACTCGGAGTTGAATCCTATAAAACCGAGACACTCACGCCT
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TGATGAAGAGTTGAGGAGTCAGGGCCGTTGCCCAATGACTCCTGAAGAGGTTGGATTGTTGCTAGCAGCTTTGGGATT
CGACAACAGCACTCGTTTATATCTTGCCTCTCACAAGGTCTATGGTGGAGGAGCCAGGATTGCTACTTTGAAGCAATT
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GGTTGGACACAGGACTTACTTGAACTTAAAGACTATAAGGCCAAACATGGCATTGATGGGTCAACTGTTCTTGAATAA
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GCCTAAACAATCCATATACACATACCCTGTTCCTGATTGCATGTGCCAAGCTTAATAGATTGCTTACAGATTAGTAGC
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GAGGGGTTTGTCCATGATGTTCTTGGTAAGAGTGCTTGATATAATAGTATAGTAGTAGGTGTCTTGTGTGAGAGATGT
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Rogue = 1528 Onward = 4

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CCCAAAGATCACTCTGTCTCATTTCCTGAGAATGTCCAAGGAGGGAAGTTGGTAGTTTGTTCGGACAGAACCGCAGTC
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TC

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Rogue =1157 Onward =5

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CACTCACACATCTCTCCCTCAAGGAATGACAAACGCCAGTCATTTGACAAAGCGAAGACGGAGGTAGCCTTTCAAGAA
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Rogue =1039 Onward =0

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CGGCCGACGACAACTGTGCCGGTAAACCGTTTAACATTCGGATCAAGTTTTCCAAAACTGAAAGCCACCTTCAACGAC
GGCGTTGTGACGGAGAAATTGAGTTTCTACGATCTTCTAGGGATTCCCGTATCTGGTTCGTTGAATGATATCAAATCT
GCTTATAAACAACTTGCGAGAAAGTATCATCCTGACGTTTCGCCACCGGACCGGGTTGAAGAGTATACGAAAAAGTTT
ATTCAGGTTCATGAGGCTTATGAGACGTTATCGGATCCTTCAAGGAGAATCATGTATGATCAGGATATGGCTAGAGGC
GTGAATCTCGCTTTCAACGCTCGGAAACGCTATAATCACTCCGATCAGGGGAATGAACAAAAAGACGAATGGAAAATC
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Rogue =866 Onward =0

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Rogue =889 Onward = 3

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Rogue =829 Onward =0

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Onward =1627 Rogue = 0

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Onward =1624 Rogue =0

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Onward =1137 Rogue =0

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Onward =1579 Rogue =1

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Onward =959 Rogue =0

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TGAATGCTTTGACATTATATCGTTTCGCAAGGCTTTGATCTTTCTGGATTGTTTGAGGAAAGGGCGACGAAGCAAGGT
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AGAAAGATTGCAGGGTTAGCTTCGAGGGTTCGAGAGAAGGGGTAAAGGGGCCGTTGAGTATCGCTGCTGAGGTATTCG
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CTTTGCAACAACGCGCGTTTTCTGATTCCGCCATTGACAAACATTCAGATAGCATTGAATCTCTGAACTTAGACACCT
GAAGAATGAATGACCTATAATAAGATAAAAAGGGTATTTTATTTTCAATGTTTTTATAGGCTGTGTATTTATGGTACT
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Annex III

>ID|Pisum_sativum_v2_Contig4801 (SSHR18)

Rogue =807 Onward =0

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Polyamine oxidase [Medicago truncatula]

Sequence ID: ref|XP_003600621.1|Length: 492

Expect: 0.0 Identities: 465/492(95%) Positives: 481/492(97%) Gaps: 0/492(0%)

```
Query  41   MDPKLFFPNKFLDGTITSLIDSQQRSVPSVIVIGAGISGIAAARILHDASFKVTLLESRD  100
            MDPKLFF N FLDGTITSLIDSQQR  PSVIV+GAGISGIAAARILHDASFKVTLLESRD
Sbjct  1    MDPKLFFSNNFLDGTITSLIDSQQRPAPSVIVVGAGISGIAAARILHDASFKVTLLESRD  60

Query  101  RLGGRIHTDYSFGCPVDMGASWLHGVCNENPLAPLIRCLGLTLYRTSGDDSVLYDHDLES  160
            RLGGRIHTDYSFGCPVDMGASWLHGVCNENPLAPLIRCLGLTLYRTSGDDSVLYDHDLES
Sbjct  61   RLGGRIHTDYSFGCPVDMGASWLHGVCNENPLAPLIRCLGLTLYRTSGDDSVLYDHDLES  120

Query  161  CMLFDIDGNKVQQQTVIEVGETFKRILEETGKVRDENPEDISVSEAISIVLDRHPELRKQ  220
            CMLFDIDG++V QQTVIEVGETFKRILEETGKVRDE+PEDISVSEAISIVLDRHP+LR+Q
Sbjct  121  CMLFDIDGHQVPQQTVIEVGETFKRILEETGKVRDEHPEDISVSEAISIVLDRHPQLRQQ  180

Query  221  GLAHEVLQWFICRMEAWFAADADMISLKSWDQEHVLSGGHGLMVQGYNPVINALAKDIDI  280
            GL+HEVLQW+ICRMEAWFAADADMISLK+WDQEHVLSGGHGLMVQGY PVINALAKDIDI
Sbjct  181  GLSHEVLQWYICRMEAWFAADADMISLKTWDQEHVLSGGHGLMVQGYKPVINALAKDIDI  240

Query  281  RLKHRVAKISNGYNKVMVTLEDGRNCVADAAIITVPIGVLKANLIEFEPRLPDWKVSAIS  340
            RL HRV KIS+GYNKVMVTLEDGRN VADAAIITVPIG+LKANLIEFEPRLPDWKVSAIS
Sbjct  241  RLNHRVTKISSGYNKVMVTLEDGRNFVADAAIITVPIGILKANLIEFEPRLPDWKVSAIS  300

Query  341  DLGVGNENKIALRFDKVFWPDVELLGVVAPTSYACGYFLNLHKATGHPVLVYMAAGRFAY  400
            DLGVGNENKIAL+FDKVFWPDVEL+GVVAPTSYACGYFLNLHKATG+PVLVYMAAGRFAY
Sbjct  301  DLGVGNENKIALKFDKVFWPDVELMGVVAPTSYACGYFLNLHKATGNPVLVYMAAGRFAY  360

Query  401  DLEKLSDESAANFVMLQLKKMFPDACEPVQYLVPHWGTDPNSLGCYSYDLVGKSMDVYDK  460
            DLEKLSDESAANFVMLQLKKMFPDACEPVQYLV HWGTDPNSLGCYSYDLVGKSMDVYDK
Sbjct  361  DLEKLSDESAANFVMLQLKKMFPDACEPVQYLVSHWGTDPNSLGCYSYDLVGKSMDVYDK  420

Query  461  LRAPLGNLFFGGEAVSLDNQGSVHGAYSAGVMAAENCQRYLWEKQGNLESLSQVSVRHET  520
            LRAPLGN+FFGGEA+SLDNQGSVHGAYSAGVMAAENCQRYLWEKQGNLESLSQVS RHET
Sbjct  421  LRAPLGNIFFGGEAMSLDNQGSVHGAYSAGVMAAENCQRYLWEKQGNLESLSQVSARHET  480

Query  521  LGTNFPLQISRI  532
            LGTNFPLQISRI
Sbjct  481  LGTNFPLQISRI  492
```

>ID|Pisum_sativum_v2_Contig5242 (SSHR19)

Rogue =785 Onward =0

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TGTTAGTAGTATCTTTGTTGTAATTGTTGCCCTGTGAAAGAAAATTTAGAGATATATGCTATCCCATATTACCCAACC
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TGTTTGCAATTATCTAAATGTTGTATCAGTATTTGTATTTGTGGAGATAATGACAAGGTGTTCTTTAATTTAGTTATG
CTTAGCA

Glycogen synthase kinase [Medicago truncatula]
Sequence ID: ref|XP_003591238.1|Length: 411
Expect: 0.0 Identities: 398/412(97%) Positives: 406/412(98%) Gaps: 2/492(0%)

```
Query  11   MASVGVAPTSGFREASGHGEIGVD-VLPEEMNDMKIRDDREMEATVVDSGNGTETGHIIV  69
            MASVGVAPTSGF+E+ G GEIGVD +LPEEM+DMKIRDDREMEATVVD GNGTETGHIIV
Sbjct  1    MASVGVAPTSGFKESLGDGEIGVDDILPEEMSDMKIRDDREMEATVVD-GNGTETGHIIV  59

Query  70   TTIGGRNGQPKQTISYMAERVVGHGSFGVVFQAKCLETGETVAIKKVLQDKRYKNRELQT  129
            TTIGGRNGQPKQTISYMAERVVGHGSFGVVFQAKCLETGETVAIKKVLQDKRYKNRELQT
Sbjct  60   TTIGGRNGQPKQTISYMAERVVGHGSFGVVFQAKCLETGETVAIKKVLQDKRYKNRELQT  119

Query  130  MRLLDHPNVVSLKHCFFSTTEKDELYLNLVLEYVPETVHRVIKHYNKLNQRMPMIYVKLY  189
            MRLLDHPNVVSLKHCFFSTTEKDELYLNLVLEYVPETVHRVIKHY+KLNQRMPMIYVKLY
Sbjct  120  MRLLDHPNVVSLKHCFFSTTEKDELYLNLVLEYVPETVHRVIKHYSKLNQRMPMIYVKLY  179

Query  190  TYQIFRALSYIHRCIGVCHRDIKPQNLLVNPHTHQVKLCDFGSAKVLVKGEPNISYICSR  249
            TYQIFRALSYIHRCIGVCHRDIKPQNLLVNPHTHQVKLCDFGSAKVLVKGEPNISYICSR
Sbjct  180  TYQIFRALSYIHRCIGVCHRDIKPQNLLVNPHTHQVKLCDFGSAKVLVKGEPNISYICSR  239

Query  250  YYRAPELIFGATEYTTAIDVWSVGCVLAELLLGQPLFPGESGVDQLVEIIKVLGTPTREE  309
            YYRAPELIFGATEYTTAIDVWSVGCVLAELLLGQPLFPGESGVDQLVEIIKVLGTPTREE
Sbjct  240  YYRAPELIFGATEYTTAIDVWSVGCVLAELLLGQPLFPGESGVDQLVEIIKVLGTPTREE  299

Query  310  IKCMNPNYTEFKFPQIKAHPWHKIFHKRMPPEAVDLVSRLLQYSPNLRCQALDALTHPFF  369
            IKCMNPNYTEFKFPQIKAHPWHKIFHKRMP EAVDLVSRLLQYSPNLRCQALD LTHPFF
Sbjct  300  IKCMNPNYTEFKFPQIKAHPWHKIFHKRMPAEAVDLVSRLLQYSPNLRCQALDCLTHPFF  359

Query  370  DELREPNARLPTGRFLPPLFNFKPHELKGVPLETLVKLVPEHARKQCPFLGL  421
            DELR+PNARLPTGRFLPPLFNFKPHELKGVP+ETL+KLVPEHARKQCPFLGL
Sbjct  360  DELRDPNARLPTGRFLPPLFNFKPHELKGVPVETLMKLVPEHARKQCPFLGL  411
```

>ID287628|p.sativum_wa1_contig23351 (SSHR20)
Rogue =775 Onward =0

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TTCTCCCTCTTCTTCTTCGCTTTACCACTACACTACCACCAA

Fructosamine kinase [Medicago truncatula]
Sequence ID: gb|KEH18974.1|Length: 319
Expect: 0.0 Identities: 292/319(92%) Positives: 305/319(95%) Gaps: 1/319(0%)

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Query  7    MSTSTCFSSLPPPSFTKTKPSPMCSMSKDPVREWILSEGKASNITKISSVGGGCINFANR  66
            MSTST FSSLP PSFTKTK  PMCSMSKDPVREWILSEGKAS ITKIS VGGGCINFANR
Sbjct  2    MSTSTFFSSLPLPSFTKTKSLPMCSMSKDPVREWILSEGKASKITKISPVGGGCINFANR  61

Query  67   YDTDVGSFFVKSNRSIGPSMFEAEALGLGAMYETGTIRVPKPYKVGPLPTGGSFIIMEFV  126
            YDTD GSFFVK+NRSIGPSMFEAEALGLGAMYETGTIRVPKPYKVG LP+GGSFIIMEF+
Sbjct  62   YDTDAGSFFVKTNRSIGPSMFEAEALGLGAMYETGTIRVPKPYKVGSLPSGGSFIIMEFI  121

Query  127  EFGGSRGDQSVLGRKLAEMHKSGKSSKGYGFEVENTIGSTPQINTWSSDWVRFYGEHRLG  186
            EFGGSR DQSVLGRKLAEMHKSGKSSKG+GF+VENTIGSTPQINTWSSDW++FYGEHRLG
Sbjct  122  EFGGSR-DQSVLGRKLAEMHKSGKSSKGFGFDVENTIGSTPQINTWSSDWIQFYGEHRLG  180

Query  187  YQLQLALDRYSDRTIFEKGQRLVKNMAPLFENVAIEPCLLHGDLWSGNVSSDKNGEPVIL  246
            YQLQLA D+YSDRTI EKGQRLV+N+ PLF+NV IEPCLLHGDLWSGN+SSDKNGEPVIL
Sbjct  181  YQLQLAFDQYSDRTILEKGQRLVENIKPLFDNVEIEPCLLHGDLWSGNISSDKNGEPVIL  240

Query  247  DPACYYGHNEAEFGMSWCAGFGGSFYNSYFEVIPKQPGFEKRRDLYMLYHYLNHYNLFGS  306
            DPACYYGHNEAEFGMSWCAGFGGSFYNSYFEVIPKQPGFEKRRDLY+LYHYLNHYNLFGS
Sbjct  241  DPACYYGHNEAEFGMSWCAGFGGSFYNSYFEVIPKQPGFEKRRDLYLLYHYLNHYNLFGS  300

Query  307  GYRSSAMSIIGDYLAYLKA  325
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Sbjct  301  GYRSSAMSIIDDYLAYLKA  319
```

>ID281115|p.sativum_wa1_contig19650 (SSHO2)
Onward =1666 Rogue =1

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ACCAACAGAACCACCATCTCCACTAACCGCAGTTGAAGA
```

BEL1-related homeotic protein [Medicago truncatula]
Sequence ID: gb|KEH41936.1 |Length: 649
Expect: 0.0 Identities: 340/377(90%) Positives: 353/377(93%) Gaps: 10/377(2%)

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Query  19   LRFKRFQKQFRCLKDAITGQIRAANKRLGEDDSFGGKIEGSRLKYVDHHLRQQRAIQQLG  78
            L  +   KQFRCLKDAITGQIRAANK LGEDDSFGGKIEGSRLKYVDHHLRQQRAIQQLG
Sbjct  281  LALQTISKQFRCLKDAITGQIRAANKSLGEDDSFGGKIEGSRLKYVDHHLRQQRAIQQLG  340
```

```
Query  79   MIHHNAWRPQRGLPERSVSVLRAWLFEHFLHPYPKDSDKHMLAKQTGLTRSQVSNWFINA  138
            MIHHNAWRPQRGLPERSVSVLRAWLFEHFLHPYPKDSDKHMLAKQTGLTRSQVSNWFINA
Sbjct  341  MIHHNAWRPQRGLPERSVSVLRAWLFEHFLHPYPKDSDKHMLAKQTGLTRSQVSNWFINA  400

Query  139  RVRLWKPMVEEMYTEEMKDQETNGSEDNKSSKNTNEDPSIKTTTPQERVPTSETESKSFN  198
            RVRLWKPMVEEMY EEMKDQE NGSEDNKSSKNT+EDPS+KT TPQERVPTSETESKSFN
Sbjct  401  RVRLWKPMVEEMYMEEMKDQELNGSEDNKSSKNTDEDPSMKTPTPQERVPTSETESKSFN  460

Query  199  SKQELAIVSVSTQSTSPIGVNVRNNSGFSFTELDGMTQASPKRTRNNDILHSPNHMKSNE  258
            SKQ++ +VSVST STSPIGVNVRNNSGFSFTELDG+TQASPKRTRN++IL SPNH+KSNE
Sbjct  461  SKQDIPMVSVSTPSTSPIGVNVRNNSGFSFTELDGITQASPKRTRNHEILQSPNHVKSNE  520

Query  259  -TENNEQISMKFGDDRQSRDGYCFMGNQTNFIAGFGQYPMEEIGRFDAEEFAPPRFSG-N  316
             T NNEQISMKFGDDRQSRDGYCFMGNQTNFIAGFGQYPMEEIGRFDAE+FA PRFSG N
Sbjct  521  TTANNEQISMKFGDDRQSRDGYCFMGNQTNFIAGFGQYPMEEIGRFDAEQFA-PRFSGNN  579

Query  317  NGVSLTLGLPHCDTLSGTHQSFMPNQNIQLGRRLEINETNEFGAINNSNSHSSAAFESIN  376
            NGVSLTLGLPHCDTLSGTHQSFMPNQNIQLGRRL+I+ETNEFG        SSAAFESIN
Sbjct  580  NGVSLTLGLPHCDTLSGTHQSFMPNQNIQLGRRLDISETNEFG-------DSSAAFESIN  632

Query  377  MQNPKRFAAQLLPDFVA  393
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Sbjct  633  MQNPKRFAAQLLPDFVA  649
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>ID|Pisum_sativum_v2_Contig5891 (SSHO6)

Onward =1427 Rogue =1

```
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AGAAAATTGAAGGTGAAAAGAGTAGTTAGAGAAATAGAAGGAGAAAAACAAATAGATGAAGTTTTAGAATATGATTGA
TTACTGTTTGAATATTTTAGCTATTTGACATTAAGTGCATATAACTTTCATTCTGGTTTCTTTTGTAACTATTACTGT
GATTTTTCTATTGATGTTTCAAAGCATTTTTTTCTTGNNN
```

Glucose 6 phosphate/phosphate translocator-like protein [Medicago truncatula]

Sequence ID: ref|XP_003594481.1|Length: 408

Expect: 0.0 Identities: 378/420(90%) Positives: 390/420(92%) Gaps: 14/420(3%)

```
Query  25   MLSLNVFPSSSS--VPFTKPNHHFSINASHTLKPNLLNRFHHESHLKKTNLSLTPTSQIH  82
            MLS NVFP+SSS  V FTKPNHHFSINAS    PNLLNRFHHES    + LS  P SQIH
Sbjct  1    MLSFNVFPTSSSSSVTFTKPNHHFSINAS----PNLLNRFHHES----SKLSFKPISQIH  52

Query  83   RSTTKLASFNGFFSYPFEFSPFSPKPRNQILKAVSDEGEVSPPSTTPKPKNLKKLALVFG  142
             STTKL+SFN F ++PFEFSP   KPRNQILKAVSDEGE+S P   PKPKNLKKLALVFG
Sbjct  53   HSTTKLSSFNRFLTHPFEFSP---KPRNQILKAVSDEGEISQP-INPKPKNLKKLALVFG  108

Query  143  FWYFQNIVFNIYNKKVLNIFSFPWLLASFQLFVGSIWMLVLWSLKLQPCPKISKPFIFAL  202
            FWYFQNIVFNIYNKKVLNIFSFPWLLASFQLFVGSIWMLVLWSLKLQPCPKISKPFIFAL
Sbjct  109  FWYFQNIVFNIYNKKVLNIFSFPWLLASFQLFVGSIWMLVLWSLKLQPCPKISKPFIFAL  168
```

```
Query  203  LGPALFHTIGHISACVSFSKVAVSFTHVIKSAEPVFSVIFSSVLGDRYPIQVWLSILPIV  262
            LGPALFHTIGHISACVSFSKVAVSFTHVIKSAEPVFSVIFSSVLGDRYPIQVWLSILPIV
Sbjct  169  LGPALFHTIGHISACVSFSKVAVSFTHVIKSAEPVFSVIFSSVLGDRYPIQVWLSILPIV  228

Query  263  LGCSLAAVTEVSFNIQGLWCALISNVGFVLRNIYSKKSLQNFKEVDGLNLYGWITILSFL  322
            LGCSLAAVTEVSFN+ GLWCALISNVGFVLRNIYSKKSLQNFKEVDGLNLYGWITILSF+
Sbjct  229  LGCSLAAVTEVSFNVGGLWCALISNVGFVLRNIYSKKSLQNFKEVDGLNLYGWITILSFM  288

Query  323  YLFPVAIFVEGSQWIPGYYKAIEAIGKPSILYVWVLVSGVFYHLYNQSSYQALDEISPLT  382
            YLFPVAIFVEGSQWIPGYYKA+EAIG PS  Y+WVLVSG+FYHLYNQSSYQALDEISPLT
Sbjct  289  YLFPVAIFVEGSQWIPGYYKALEAIGTPSTFYIWVLVSGLFYHLYNQSSYQALDEISPLT  348

Query  383  FSVGNTMKRVVVIVSSVLVFRNPVRPLNGLGSAIAILGTFLYSQATAAKKAKKIEGEKSS  442
            FSVGNTMKRVVVIVSS+LVFRNPVRPLNGLGSAIAILGTFLYSQATAAKKAKKIEGEKSS
Sbjct  349  FSVGNTMKRVVVIVSSILVFRNPVRPLNGLGSAIAILGTFLYSQATAAKKAKKIEGEKSS  408
```

>ID|Pisum_sativum_v2_Contig1549 (SSHO7)

Onward =1323 Rogue =2

```
GAAAGTGACATTCTGTACTTTGTTTTTGAGTACATGGAATGCAATCTATACCAACTTATGAAAGACAGGGAAAAATTG
TTCTCTGAAAGTGAAATTAGAAACTGGTGTTTTCAAGTTTTCCAAGGTCTGGCATACATGCACCAGCGTGGATACTTC
CACCGCGACCTAAAACCTGAAAACTTGCTGGTCACCAAGAATACTATCAAAATTGCTGATTTCGGCCTAGCACGAGAG
ATCAACTCACAACCACCCTATACTGAGTATGTCTCCACACGGTGGTATCGTGCTCCTGAAGTGCTGCTTCAGTCTTAT
ATCTATAATGCCAAAGTTGACATGTGGGCAATGGGTGCTATAATGGCCGAGCTCTTCTCTCTACGACCTCTTTTTCCC
GGAGCCAGTGAGGCAGATGAGATCTACAAAATATGTGGTGTGATAGGCAGTCCAACCACTGAATCATGGGCTGTTGGA
CTCAAACTTGCAAGAGATATTAATTATCAGTTTCCACAGCTTGCTGGCGTAAACCTTTCTTCATTGATCCCATCTGCA
AGTGATCATGCAATCAGCCTTATCCAATCACTTTGCTCTTGGGATCCCTGCAAGAGGCCAACAGCTTTAGAGGCCCTT
CAACATCCTTTCTTCCAGAGTTGCTTTTACATTCCTCCATCCCTTCGCTCCAGAGCAATAGCGAGAACCCCTCCACCT
GCAACTAGGGGATCACTGGATCAGCAAGGGGTCAAGAGATATCCCGGTGCTTTACCCAACTCAAAACTCACCAATTAT
TTTCCATCTCCAAAGTTACAGCCTTCTTCAGGTGTGCAACGGAAGTTGGATATGGTAAATCAGGATGACATTCAGAAT
GATAAATCAATGAAGACTACTACACAATTGAAGTATCGACAACCAGGAAAGGACAGTCAAACTTCTATAAACAAAGGA
AGATCTACACGAGGTGCTTTAGAAACAGCTGAGAGGTTGGCCAATATGTCTATTGGCACTCGAAGACAGTCCATGGAG
CAACCCCGTGCTCCTCCCATGAAGGCTGGAGTCAATTGGAGTTCTCCCATGAAGGCTGGAGTCAATTGGAGTTCCGAA
TCTCCTAACTTCATGCTCAGACCTGCACCACAGATCTCAACTGGGAGAACGTACCCTAGAAAAGTTGCTGGATGAGAA
GAACAGCCTCTATATTATTGCGACTATCCTAGTTTAAACAATATATATTTGGGTTTTGCTCGTGTTATGTTTACTGCA
AAACTAGTTTGCCCGTGTCATTAATAATCGTTTATACTGTGTGTTGTGGGACAAACACGCATGAGCTGATACTTAAAT
AAGTCTTTCTTAGCTTATGAACTTTGATGTGTGCGGTGTTTATAAATTGCCTTCTTGGTCAATAAACAATAATTTAGT
AATGTTGCTGGTCTTAATGACTACACCTGTACTTAATGGTTGAATTGTGAAGGT
```

Serine/threonine protein kinase ICK [Medicago truncatula]

Sequence ID: ref|XP_003612616.1|Length: 449

Expect: 0.0 Identities: 378/420(90%) Positives: 390/420(92%) Gaps: 14/420(3%)

```
Query  1    ESDILYFVFEYMECNLYQLMKDREKLFSESEIRNWCFQVFQGLAYMHQRGYFHRDLKPEN  60
            ESDILYFVFEYMECNLYQLMKDREKLFSE EIRNWCFQVFQGLAYMHQRGYFHRDLKPEN
Sbjct  71   ESDILYFVFEYMECNLYQLMKDREKLFSEGEIRNWCFQVFQGLAYMHQRGYFHRDLKPEN  130

Query  61   LLVTKNTIKIADFGLAREINSQPPYTEYVSTRWYRAPEVLLQSYIYNAKVDMWAMGAIMA  120
            LLVTK+ IKIADFGLAREINSQPPYTEYVSTRWYRAPEVLLQSYIY++KVDMWAMGAIMA
Sbjct  131  LLVTKDVIKIADFGLAREINSQPPYTEYVSTRWYRAPEVLLQSYIYSSKVDMWAMGAIMA  190

Query  121  ELFSLRPLFPGASEADEIYKICGVIGSPTTESWAVGLKLARDINYQFPQLAGVNLSSLIP  180
            ELFSLRPLFPGASEADEIYKICGVIG+PTT+SWA GLKLARDINYQFPQLAGVNLS+LIP
Sbjct  191  ELFSLRPLFPGASEADEIYKICGVIGNPTTDSWADGLKLARDINYQFPQLAGVNLSALIP  250

Query  181  SASDHAISLIQSLCSWDPCKRPTALEALQHPFFQSCFYIPPSLRSRAIARTPPPA-TRGS  239
            SASDHAISLIQSLCSWDPCKRPTA EALQHPFFQSCFYIPPSLRSRA+ARTPPPA TRG+
Sbjct  251  SASDHAISLIQSLCSWDPCKRPTASEALQHPFFQSCFYIPPSLRSRAVARTPPPAGTRGA  310

Query  240  LDQQGVKRYPGALPNSKLTNYFPSPKLQPSSGVQRKLDMVNQDDIQNDKSMKTTTQLKYR  299
            LDQQGVKRYPGAL +SK TNYF SPK+QPSSGVQRKLDMVNQ+ I+N+KSMKTTTQ KYR
Sbjct  311  LDQQGVKRYPGALHSSKPTNYFSSPKVQPSSGVQRKLDMVNQEGIKNEKSMKTTTQSKYR  370

Query  300  QPGKDSQTSINKGRSTRGALETAERLANMSIGTRRQSMEQPRAPPMKAGVNWSSPMKAGV  359
             PGK+S TS+ KGR+  G  ETAERLANMSIG RRQSM QPR PPM          KAGV
Sbjct  371  HPGKESPTSVIKGRTIHGISETAERLANMSIGNRRQSMGQPRPPPM----------KAGV  420
```

```
Query  360  NWSSESPNFMLRPAPQISTGRTYPRKVAG  388
            NWSSESPNFMLRPAPQI TGRTYPRKVAG
Sbjct  421  NWSSESPNFMLRPAPQIPTGRTYPRKVAG  449
```

>ID|Pisum_sativum_v2_Contig4284 (SSHO8)

Onward =1135 Rogue =0

TGGAAGATGCTAAATTTGGAACCTTATTTCACTTTAATTCTTATACCCTTTAACCAAAAAGTAACCAAAAAATCTACT
TAAGTCATCATTCCTTTCCATTGCCATTAAGCACCAACAAGCAAATCCTATAACAACCAAACCAAGCTACCATTACAA
ACCCTCTTTGAATTGCACTCCaTCTCAAACATGTAACGTAGCTTTCACTCCTCTACCCCATATGGAACCATTATTTCT
TGAGAAAACTTGGGGTAGTGGAGAAAGTGGATGGTGTGTGTGTGTTAATGGTTTCCAATTTCCATGCTCCTTTTCTTT
CATGACTCTATGTTAAAGATTGATGGGAACGAACCTTCTTTGTAGATGGGAAATTCTCTTTGGAAAGGGCAACTTCGA
TCCTTTGGCATTTTTCAATTCCTTCATCTTGTGAGATTTGATGAAGCTTTCATAATGGAAACACTTCTATTGTGTAAT
ATGTATTGTACTCTCTTCTTTTCCTATAATCTTTTTTTGTGTGTGTTGTTATGTGATGATAACTTCTAAGTTGTAACT
GCAAGCTATGTTTGTAAGACATGCCTACTATAGACTATAGTATTAGTAGGTATATCCAATAAATGTACGTTTACTTAT
GCCACTGGCCAAATATTTTATTATTGCTGTTGTTTCTAAATCTAT

The Contig4284 does not translate in a viable protein

>ID61637|Pisum_sativum_v1_Contig2226 (SSHO10)

Onward =1021 Rogue =0

CATCATCTCTTTGGTAACTTCCTGGACCTCTAAGCAATTCTTCTGAGCCCTTGGACACAACACCTTTCATTATTATAG
TCTTGTGTGTCCCCGAAATCAGTTCCTCGTAATCAGTGTCCCCGGTTTCTCCGAGGATCACATACATGTTTGCGACGT
TTAGTCTCCATCGGACAAATAGATACCTTAGTGCTTGAGCTCTAGATGCAAGAAGAGGAATAACATGTATTCTAGAAG
ATCCTCTACAATACATAGGATGACATCTTAAACCTCTCATCCTAAGCTTTTGCCTCAAGTCATCAACTTTCTTCGCCT
TACTAAGATCATTTACTTTGTATGAAATACAATGCGCGTTACTTGATTTCGAATCTTCCTCAATAGGACTAATTGTTT
TTCCATGATTTTCTTCTCCATCGGAGGCATTCATAAGTTTACGAATAGTATTCTTCAAACCTTCGACGCCCCAACGAT
AATCTATATGCACGTCATAATCATGATCGGGCAAAAGCTTTCCGTCTTCGGTATGAACACCGGGGGTAATAAACTTCA
CTCCCACTGCTTAC

Sucrose-phosphate synthase [Medicago truncatula]

Sequence ID: ref|XP_003617418.1|Length: 1058

Expect: 1e-106 Identities: 161/183(88%) Positives: 169/183(92%) Gaps: 0/183(0%)

```
Query  3     GVKFITPGVHTEDGKLLPDHDYDVHIDYRWGVEGLKNTIRKLMNASDGEENHGKTISPIE  62
             G +   PGVHTEDGKLLPD DY VHIDYRWGVEGLKNTI KLMNAS+GEE +G   SP+E
Sbjct  843   GSEVYYPGVHTEDGKLLPDQDYAVHIDYRWGVEGLKNTICKLMNASNGEETNGIATSPLE  902

Query  63    EDSKSSNAHCISYKVNDLSKAKKVDDLRQKLRMRGLRCHPMYCRGSSRIHVIPLLASRAQ  122
             ED KSSNAHCISYK+ND SKA+KVDDLRQKLRMRGLRCHPMYCRGSSR+HVIPLLASRAQ
Sbjct  903   EDLKSSNAHCISYKINDPSKARKVDDLRQKLRMRGLRCHPMYCRGSSRMHVIPLLASRAQ  962

Query  123   ALRYLFVRWRLNVANMYVILGETGDTDYEELISGTHKTIIMKGVVSKGSEELLRGPGSYQ  182
             ALRY FVRWRLNVANMYVILGETGDTDYEE+ISGTHKTIIMKGVVSKGSEELLRGPGSYQ
Sbjct  963   ALRYFFVRWRLNVANMYVILGETGDTDYEEMISGTHKTIIMKGVVSKGSEELLRGPGSYQ
1022

Query  183   RDD  185
             RDD
Sbjct  1023  RDD  1025
```

>ID266692|p.sativum_wa1_contig30745 (SSHO12)

Onward =966 Rogue =0

AGCTACCACTTCTGATTTCCTGCAGAACATGCAAAGCAGATATTGTACTCTTCAAATAGAGACTCTTCTGCATTTCAG
CCATCTCCGTCGGTATAAAAGGATCATCTCTGTTCGTAGCATATGTCGAAGTAGAAGGATCGTCTCCGCCCGGTTCAG
CTGACTCGCCAAAGGGGAAAAGACCATCCAGCATTGTTTCACACTGTTTTATAAGCTTGTAGAGCAGGTCAGTGGTAA
AGAACGGTTCTTGTAAAACCTTCTGAATGAATGGCAACCGAATGAGAGCTCCTGTTCTCTTGTCATACTTCTTTAGAA
TTTTCACTAGTCCTGTGTAATTAAGAGCACTATAGTTTTCAAGCAAAACCATTTCTCCATGGAAATCTACGATATCCT
TGTGAATTTGCATCATTTGTTCGTTAGAACCCTTTCCTTTGGCCACACAATCTTGTAACTCCTTGAATCTTATGATGC
ACTCTTCCTCTTTCTCAACGAAGAAACAATTGAATTTGTGGAGTTCATCTTCGAGGGAATTCCGGAAATCGGTTTCTT
CATTTGACATTTCTCCGTCGTCGATCCTAGCTCTTTTGTTCGGGCGTTCATCGGAGGTTGGTTGAACAAGCTTCAATT

TCTTCTTGAGTAGCTTGTAAGAGAGAAATTTGTCACGCCATTGAGGAACGGTTTTCTCGATCTGGTTGTTGAGACTCT
TTCCGAATTTCATCGTTTAGGTTAAAGATTAGAAGATGGATTGGATTGGATTGAATTC

PREDICTED: SPX domain-containing protein 2-like [Glycine max]
Sequence ID: ref|XP_003549761.1|Length: 295
Expect: 2e-114 Identities: 181/264(69%) Positives: 205/264(77%) Gaps: 28/264(10%)

```
Query  2    MKFGKSLNNQIEKTVPQWRDKFLSYKLLKKKLKLVQP-----TSDERPNKRARIDDG---  53
            MKFGKSL++QIEKT+P+WRDKFLSYK LKKKLK   P      +DERP KR + D G
Sbjct  1    MKFGKSLSSQIEKTLPEWRDKFLSYKELKKKLKQFDPPAPASAADERPGKRLKTDAGNAD  60

Query  54   --------EMSNEETDFRNSLEDELHKFNCFFVEKEEECIIRFKELQDCVAKGKGSNEQM  105
                    +MS EE+DFRN LE+EL KFN FFVEKEEE IIR KELQD VA+ KGS E+M
Sbjct  61   ADAVSDASDMSKEESDFRNLLENELDKFNTFFVEKEEEYIIRLKELQDSVAQVKGSREEM  120

Query  106  MQIHKDIVDFHGEMVLLENYSALNYTGLVKILKKYDKRTGALIRLPFIQKVLQEPFFTTD  165
            M+IHK+IVDFHGEMVLLENYSALNYTGLVKILKKYDKRTGALIRLPFIQKVLQ+PFFTTD
Sbjct  121  MKIHKEIVDFHGEMVLLENYSALNYTGLVKILKKYDKRTGALIRLPFIQKVLQQPFFTTD  180

Query  166  LLYKLIKQCETMLDGLFPFGESA--------EPGGDDPSTSTYATNRDDPFIPTEMAEMQ  217
            LLYKL+K+CETMLD LFP  + A        +  G DPSTST  T  D   IP E+AE++
Sbjct  181  LLYKLVKECETMLDHLFPVNDPAPVSTETTPQAEGFDPSTST-TTKSDGLVIPKELAEIE  239

Query  218  --KSLYLKSTISALHVLQEIRSGS  239
              +SLY+KST+SALHVLQEIRSGS
Sbjct  240  YMESLYMKSTVSALHVLQEIRSGS  263
```

>ID|Pisum_sativum_v2_Contig7337 (SSHO13)
Onward =1041 Rogue =1

```
GAGGACGCGTCGCGTACACCTCACATCTTCCCATTTCCTTTCTCTACTTAACCATCCTCTTTCCTTCTTCCATTCCAT
TGCATACTCAACAAGCAACCATTTCATCCTCCTCGAGATCTGTGCTTCCTCTTCTTCCTTCTCTCCCTTCTTCCTTAA
CTGTCGGCTGAAGATATGGCTCCACCAATTGAGACCCCAAACAAGGTTCAATCCTCGAATTATACCTCACCTCCACCT
CTTAATGAGAGGATCCTTTCATCCTTGACAAGGAGATCTGTTGCTGCACACCCTTGGCATGATCTTGAAATAGGCCCT
GAAGCTCCCAAGATCTTCAACTGTGTGGTTGAAATTGGGAAAGGAAACAAGGTGAAATATGAACTTGACAAAAAAACC
GGACTTATTAAGGTTGACCGTGTGCTTTACTCATCAGTTGTGTATCCTCACAACTATGGGTTTGTCCCCCGCACTATT
TGTGAGGATGGTGATCCCATCGATGTCTTGGTCATTATGCAGGAGCCAGTTCTTCCAGGATGCTTTCTTCGGGCCAAA
GCTATTGGACTCATGCCTATGATTGATCAGGGTGAGAAAGATGACAAGATAATTGCTGTCTGTGCTGATGATCCCGAG
TATAGGCATTTCAATGATATCAAGGAGCTCCCTCCACATCGTTTAGCTGAAATTCGTCGTTTTTTTGAAGATTACAAG
AAGAACGAGAACAAGGAAGTTGCAGTAAATGACTTTCTCCCTGCCTCATCTGCCTATGAAGCGATCGAGCATTCCATG
ACCTTGTATGCGGACTACGTGGTGGAGAGCTTGAGGCGGTAGCTTTGATGGATATGGGATTTTGTTTGGTCGTGAAGA
TATATTTTGAAGGCTGCCATGCCATGCCATTTTATAACTAAGATTACGACTGAAAAAGATATTTTGTATCGTATTACT
GTCATATTCCTATAGCATGTACATTTGTCTCACAAAGGACAAGTAACTACACATGCATATCCTACACACATATATTTT
GCTTGACTTTTATTTTTAAATGATATATTCGTTTATTAATATTCAAATCTGTTTTAATATCATTTTTGCCTCATTTTA
TCTTCTAAGACGTGTGTTCTTCTGGACAAACATTGCTGCCATTATTTAGTGGGGTTCACATCTAAACCAATTTTTAA
```

Soluble inorganic pyrophosphatase [Medicago truncatula]
Sequence ID: gb|KEH42358.1|Length: 248
Expect: 2e-155 Identities: 210/219(96%) Positives: 218/219(99%) Gaps: 0/219(0%)

```
Query  3    AEDMAPPIETPNKVQSSNYTSPPPLNERILSSLTRRSVAAHPWHDLEIGPEAPKIFNCVV  62
            AE+MAPPIETPNK+ ++NYTSPPPLNERILSSLTRRSVAAHPWHDLEIGPEAPKIFNCVV
Sbjct  30   AEEMAPPIETPNKIPTANYTSPPPLNERILSSLTRRSVAAHPWHDLEIGPEAPKIFNCVV  89

Query  63   EIGKGNKVKYELDKKTGLIKVDRVLYSSVVYPHNYGFVPRTICEDGDPIDVLVIMQEPVL  122
            EIGKGNKVKYELDKKTGLIKVDRVLYSSVVYPHNYGF+PRTICEDGDPIDVLVIMQEPVL
Sbjct  90   EIGKGNKVKYELDKKTGLIKVDRVLYSSVVYPHNYGFIPRTICEDGDPIDVLVIMQEPVL  149

Query  123  PGCFLRAKAIGLMPMIDQGEKDDKIIAVCADDPEYRHFNDIKELPPHRLAEIRRFFEDYK  182
            PGCFLRAKAIGLMPMIDQGEKDDKIIAVCADDPEYRH+NDIKELPPHRLAEIRRFFEDYK
Sbjct  150  PGCFLRAKAIGLMPMIDQGEKDDKIIAVCADDPEYRHYNDIKELPPHRLAEIRRFFEDYK  209
```

```
Query  183  KNENKEVAVNDFLPASSAYEAIEHSMTLYADYVVESLRR  221
            KNENKEVAVNDFLP+SSA+EAIEHSMTLYADYVVESLRR
Sbjct  210  KNENKEVAVNDFLPSSSAFEAIEHSMTLYADYVVESLRR  248
```

>ID291839|p.sativum_wa1_contig18536 (SSHO15)

Onward =869 Rogue =0

```
TTTTTTAATATATGATAAAGTTACAAATATAATTCACCATTTTACAAGTACCAGACCTACCTTCAAAGGATACAAAAG
GCAAGCAGGTCTTTAGAATGACCAGCTTAAAGTTGACAGCCCTTATAAATGCTAAATTTTTCAACAGATACATTCTCA
TGTAATTAACATTGCCAAATGTTATGTGAAGCAAGGTATTAATGGGGCTGCCGGGCACAGGCTTTTTCTCGGATATTT
ATTTCTGTATAGTAAGTTAAAATCCTTGACTTATAAATGGAATGTCATTCAAACTACTTGAATATATGACTTCATTTC
CCAACACTTGAAAGGCTCCTGATTTCATTTATCAGTCTTGAGAAAGCAGGGAAAGTCAAATTAATACATCTTCGGAAA
CAATGTGCGATCCAAAGTTCAAGAGAGAAAGAAGTCTACATATGTGGTTTTTCTCAAACTTGCTCATCAGGCTCCTTT
TTTCTTTTGGTCTTTTATAGACTTGATCTCTTTAGTAAGCATTCTTGGAGCTATGGCCATAGCCATTAGCTCTTGGAA
GAGCAGCTTACAAGCATAAGGAATGTAAACCTGAACAATGTCAGTTTTGTTCTTGCAACCCCTACACTCGAAAGAATT
CTTCTTCAGATTTGCAATAGCTATTAACCCACAGCGTTCGCATACATGGACCCTATATGCATCACTTTGATCAAACAA
TCTTTCCTTTAGGAAGTGAGCAGCTCCATGGGCTATCATGCAATCACGTTCCATCTCTCCAAACCTAAGACCACCGTC
TCGTGACCGCCCCTCAGCAGGTTGCCTTGTAAGAATCTGCACAGGACCTCGTCCACGAGAATGGATCTTGTCGTCAAC
CATATGCTTCAACCTTTGGTAATAAGTAGGGCCAAGGAAAATCATTGCACTGAGACGCCTCCCTGTATGACCATTGTA
CATTGTCTCAAAACCACGCATTTGGTATCCACATTTGTGTAGAGCTTTGCTGATATTATCCACTGTAACATCGGTAAA
AGGAGTCGCATCTCCTTCTTTTCCCATGTGGGCTGCAACCTTCCCCATAATACACTCAATAAGCTGACCAATTGTCAT
TCGAGAAGGTATAGCATGTGGATTGACAATAATATCAGGGGTGATGCCCTCCGCAGTCCAGGGCATGTCTTCTTGTGT
ATAAGTCATACCAACTGTCCCCTTCTGACCATGCCTACTACTGAATTTGTCACCAATCTGTGGTATTCGAACGGATCT
CACCCTTACTTTAACGAATCTCAACCCATCAGCATTTGTTGTCAACAGAACTTGATCAACGATTCCAGTTTCACTGTG
GCGTAAACTTATGCTGTGATCACGTTTTGAGTAACGAGCAGCTTGTCCTTGAGCCTCCTCCTGAGATAAAGGAGTGGT
CTTTCCAATAATAACATCCTCACCAGACACTCTGGTGCCAGGTGGAGCAAGGCCATCGTCGTCCAACTTATCGTAAGA
ACCATGCCTCATACCCATGGTGTTAGCTCTATCCGGCCGACCAAAATCTTCTTTGACAAGAGTTCCCATTTTCTTCTC
TTCATCTCTATATGAACGGAAAAACAGAGACCGAAAGAAGCCACGGTCTATTGATGATTGGTTCATGATGACAGAATC
TTCTTGATTATAACCAGAATAACATGAAATGGCCACAATGGCATTAATTCCAGCAGGTAGTTGACGAAAATGAAGATG
CTCCATGGCTCTCGTAGTGACCAATGGCTTTTGAGGATAGTACAATACATAGGCCAAGGTATCCATTCGAAACTGGTA
GTTGGTAACATAAATTCCCATTGCTTGTTTTCCCATTGCAGACTGATACGTGTTACGAGGAGACTGGTTGTGATCGGG
AAATGGTATGATGGAAGCACATACACCCAAAATCAGTGATGGATGGATTTCACAGTGAGTGTAAGTATCAGAATATGC
TTCTTCTGGATTGAGCCTTGCTTGAACAAGATCATTAATGGTCATGGAAATCATAGTTGTCTCTTCCTCTTCTGTGTC
AATATATTCTATAAATCCCTTGGACACAAGATCATGCCAACCACCATCTTCGGGGGATTCCCTTTGTTGCAAAGAATG
AATGTC
```

DNA-directed RNA polymerase [Medicago truncatula]

Sequence ID: ref|XP_003589372.1|Length: 1213

Expect: 0.0 Identities: 547/551(99%) Positives: 549/551(99%) Gaps: 0/551(0%)

```
Query  1     DIHSLQQRESPEDGGWHDLVSKGFIEYIDTEEEETTMISMTINDLVQARLNPEEAYSDTY  60
            DIHSLQQRESPEDGGWHDLVSKGFIEYIDTEEEETTMISMTINDLVQARLNPEEAYSDTY
Sbjct  663   DIHSLQQRESPEDGGWHDLVSKGFIEYIDTEEEETTMISMTINDLVQARLNPEEAYSDTY  722

Query  61    THCEIHPSLILGVCASIIPFPDHNQSPRNTYQSAMGKQAMGIYVTNYQFRMDTLAYVLYY  120
            THCEIHPSLILGVCASIIPFPDHNQSPRNTYQSAMGKQAMGIYVTNYQFRMDTLAYVLYY
Sbjct  723   THCEIHPSLILGVCASIIPFPDHNQSPRNTYQSAMGKQAMGIYVTNYQFRMDTLAYVLYY  782

Query  121   PQKPLVTTRAMEHLHFRQLPAGINAIVAISCYSGYNQEDSVIMNQSSIDRGFFRSLFFRS  180
            PQKPLVTTRAMEHLHFRQLPAGINAIVAISCYSGYNQEDSVIMNQSSIDRGFFRSLFFRS
Sbjct  783   PQKPLVTTRAMEHLHFRQLPAGINAIVAISCYSGYNQEDSVIMNQSSIDRGFFRSLFFRS  842

Query  181   YRDEEKKMGTLVKEDFGRPDRANTMGMRHGSYDKLDDDGLAPPGTRVSGEDVIIGKTTPL  240
            YRDEEKKMGTLVKEDFGRPDRANTMGMRHGSYDKLDDDGLAPPGTRVSGEDVIIGKTTPL
Sbjct  843   YRDEEKKMGTLVKEDFGRPDRANTMGMRHGSYDKLDDDGLAPPGTRVSGEDVIIGKTTPL  902

Query  241   SQEEAQGQAARYSKRDHSISLRHSETGIVDQVLLTTNADGLRFVKVRVRSVRIPQIGDKF  300
            SQEE QGQAARYSKRDHSISLRHSETGIVDQVLLTTNADGLRFVKVRVRSVRIPQIGDKF
Sbjct  903   SQEEQQGQAARYSKRDHSISLRHSETGIVDQVLLTTNADGLRFVKVRVRSVRIPQIGDKF  962

Query  301   SSRHGQKGTVGMTYTQEDMPWTAEGITPDIIVNPHAIPSRMTIGQLIECIMGKVAAHMGK  360
            SSRHGQKGTVGMTYTQEDMPWT EGITPDIIVNPHAIPSRMTIGQLIECIMGKVAAHMGK
Sbjct  963   SSRHGQKGTVGMTYTQEDMPWTVEGITPDIIVNPHAIPSRMTIGQLIECIMGKVAAHMGK
1022
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Query  361   EGDATPFTDVTVDNISKALHKCGYQMRGFETMYNGHTGRRLSAMIFLGPTYYQRLKHMVD  420
             EGDATPFTDVTVDNISKALHKCGYQMRGFETMYNGHTGRRLSAMIFLGPTYYQRLKHMVD
Sbjct  1023  EGDATPFTDVTVDNISKALHKCGYQMRGFETMYNGHTGRRLSAMIFLGPTYYQRLKHMVD
1082

Query  421   DKIHSRGRGPVQILTRQPAEGRSRDGGLRFGEMERDCMIAHGAAHFLKERLFDQSDAYRV  480
             DKIHSRGRGPVQILTRQPAEGRSRDGGLRFGEMERDCMIAHGAAHFLKERLFDQSDAYRV
Sbjct  1083  DKIHSRGRGPVQILTRQPAEGRSRDGGLRFGEMERDCMIAHGAAHFLKERLFDQSDAYRV
1142

Query  481   HVCERCGLIAIANLKKNSFECRGCKNKTDIVQVYIPYACKLLFQELMAMAIAPRMLTKEI  540
             HVCERCGLIAIANLKKNSFECRGCKNKTDIVQVYIPYACKLLFQELMAMAIAPRMLTKE+
Sbjct  1143  HVCERCGLIAIANLKKNSFECRGCKNKTDIVQVYIPYACKLLFQELMAMAIAPRMLTKEV
1202

Query  541   KSIKDQKKKGA  551
             K+IKDQKKKGA
Sbjct  1203  KAIKDQKKKGA  1213
```